European Journal of Pharmacology 661 (2011) 118–123

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

Effect of compound IMMLG5521, a novel coumarin derivative, on carrageenan-induced pleurisy in rats

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ARTICLE INFO

Article history:
Received 10 January 2011
Received in revised form 15 March 2011
Accepted 5 April 2011
Available online 27 April 2011

Keywords:
Coumarin derivative
Pleurisy
Inflammation
NF-κB

ABSTRACT

Accumulative evidences have showed that some coumarin derivatives have significantly anti-inflammatory effects. To investigate the potential anti-inflammatory effect of compound IMMLG5521, a novel coumarin derivative, carrageenan-induced pleurisy model in rats was employed. The results showed that IMMLG5521 (5, 10 and 20 mg/kg) exhibited anti-inflammatory effects, reducing pleural exudate formation, decreasing total number of inflammation cells and polymorphonuclear leukocytes infiltration, attenuating histological injury and reducing TNF-α, IL-1β, MIP-2 and IL-8 release. Further investigation revealed that the compound may exert its anti-inflammatory effect via inhibiting nuclear translocation of NF-κB in inflammatory cells collected from pleural exudates. Taken together, the present results suggested that IMMLG5521 inhibited acute inflammation in carrageenan-induced pleurisy model that could be, in part, related to a reduction of release of inflammatory factors, another part may be related to an inhibition of NF-κB activation.

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1. Introduction

Inflammation is a complex phenomenon composed of humoral and cellular reactions with several key inflammatory mediators involved. Acute inflammation is characterized by neutrophil activation, adhesion and migration through endothelial vascular cells and extracellular matrix to inflammatory sites. Increased vascular permeability can be observed, which leads to exudation of fluid rich in plasma proteins into the injured tissues. Exudation, which is a consequence of increased vascular permeability, is considered as a major feature of acute inflammation. Inflammation often responds to severe lesions while excessive inflammation reactions would also damage normal tissue (Lunardelli et al., 2006). During acute inflammation, serum proteins and leukocytes migrate to areas of damaged tissue (Paul et al., 2009).

Coumarins represent a vast family of compounds which were naturally found in plants (Hoult and Payá, 1996). It has been already reported that several coumarin derivatives have significantly anti-inflammatory and antioxidant activities (Fylaktakidou et al., 2001; Kontogiorgis and Hadjipavlou-Litina, 2003; Nicolaides et al., 2004). Thus, coumarin derivatives could be particularly effective in the treatment of high protein oedemas (Piller, 1975; Piller and Casley-Smith, 1975; Fylaktakidou, et al., 2004). It was reported that some coumarins possessed antioxidant capacity scavenging superoxide anion radicals (Payá et al., 1992) and some coumarins could inhibit both the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism (Hoult et al., 1994; Grimm et al., 2006).

IMMLG5521 is a 3-piperazine substituted coumarin compound. We have already reported several 3-piperazine substituted coumarin compounds, of which compound 41 could lessen the asthmatic pathologic changes, collagen deposition in the lung tissues and decrease the accumulation of EOSs in the BALF in human CKLF1-transfected mice (Li et al., 2010). Based on this research, derivation compounds of compound 41 were prepared and a pharmacological screening was conducted. IMMLG5521 showed better anti-inflammatory activity in vitro. In order to further evaluate anti-inflammatory effect of IMMLG5521 in vivo, carrageenan-induced pleurisy model, a well-established experimental model of inflammation, was adopted in the present study.

2. Materials and methods

2.1. Chemicals

The compound IMMLG5521 was synthesized in our laboratory. The compound IMMLG5521 was suspended in 0.5% sodium carboxymethyl cellulose and administrated p.o. at a volume of 10 ml/kg. The compound IMMLG5521 structure shown in (Fig. 1B).

2.2. Animals

Male Sprague–Dawley rats aged nine weeks (230 ± 10 g) were housed in groups of five per cage at a temperature of 23 ± 1 °C with a

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doi:10.1016/j.ejphar.2011.04.024

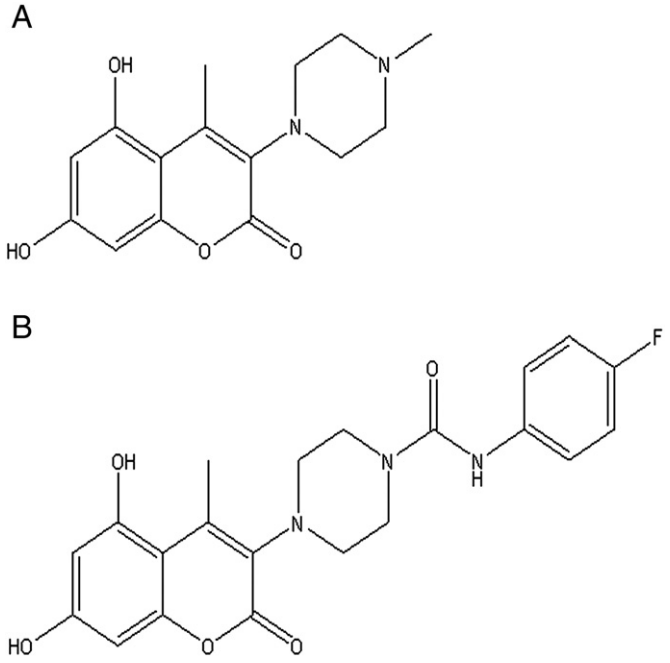

Fig. 1. Chemical structures of 41 (A) and IMMLG5521 (B).

12 h light–dark cycle (light on 7 a.m.–7 p.m.), and had free access to the food and water. All experiments were performed in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and Chinese Academy of Medical Sciences(Beijing, China).

2.3. Carrageenan-induced pleurisy

The rats were divided into IMMLG5521 (5, 10 and 20 mg/kg), indomethacin (IND 2 mg/kg), Sham and model animals group. IMMLG5521 at 5, 10 and 20 mg/kg body weight and indomethacin at 2 mg/kg body weight were administered orally once everyday and lasted for 4 days. Sham and model animals were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution. The pleurisy was induced by injection of 0.3 ml of sterile saline solution containing carrageenan (1%) into the right pleural space of animals under anesthesia 30 min after the last administration. Sham animals were injection 0.3 ml of sterile saline solution. The animals were sacrificed 4 h after carrageenan injection. The chest was carefully opened and the pleural cavity was rinsed with 2 ml of saline solution with heparin (5 U/ml). The exudate was removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume recovered. The exudate volumes were measured and then the samples were centrifuged at 800 *g* for 10 min and cell pellet resuspended in phosphate buffer saline. The number of leucocytes in the exudate was counted with optical microscope by cell counting chamber after Giemsa staining. The supernatant was used to determine TNF-α, IL-6, MIP-2 and IL-8 by ELISA and MPO.

2.4. Histological examination

Lung tissues were taken 4 h after injection of carrageenan. The tissues were fixed in 10% formalin for a week at room temperature, dehydrated and subsequently embedded in paraffin blocks. Sections were stained with hematoxylin-eosin. All sections were studied using light microscopy.

2.5. Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity in the supernatant of centrifuged exudates, an index of polymorphonuclear leukocyte accumulation was assayed by using a colorimetric, commercial kit (Nanjing Jiancheng Institute of Biological Engineering, China). MPO activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37 °C and was expressed in U/l.

2.6. TNF-α and IL-1β assay

TNF-α and IL-1β levels in the supernatant of centrifuged exudates were evaluated by using a colorimetric, commercial ELISA kit (NeoBioscience Technology Company, Beijing, China).

2.7. MIP-2 and IL-8 assay

MIP-2 and IL-8 levels in the supernatant of centrifuged exudates were evaluated by using a colorimetric, commercial ELISA kit (Westang Biotechnology, Shanghai, China).

2.8. Western blotting analysis

Cells harvested 4 h after injection of carrageenin or saline were used to extract cytosolic and nuclear protein. Antibodies against to NF-κB (p65), IκB-α, and β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA) were used as indicated by the manufacturer's instructions. The cytosolic and nuclear protein were extracted for immunoblot according to manufacturer kits (Applygen Technologies Inc., Beijing, China). Equal amounts of protein from cytosolic or nuclear fractions were analyzed by 10% SDS-PAGE electrophoresis and blotted onto PVDF membrane (Millipore, MA). The membranes were blocked in 3% BSA in Tris-buffer saline for 1 h and then incubated overnight with the IκB-α polyclonal antibody, and NF-κB (P65) monoclonal antibody at 4 °C. After incubation with the peroxidase-conjugated goat antimouse IgG and goat antirabbit IgG, the bands were visualized by ECL and quantified by using image analysis software.

2.9. Data analysis

All values are expressed as mean ± deviation (S.D.). The statistical significance of differences between the groups was analyzed by ANOVA. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of IMMLG5521 on carrageenan-induced pleurisy

Carrageenan injection into pleural cavity of animals induced exudate volume increased to 1.25 ± 0.33 ml/rat ($P<0.01$) (Fig. 2A), cell migration to $89.2 \pm 19.6 \times 10^6$ cells/rat ($P<0.01$) (Fig. 2B) and the number of neutrophils increased to $58.6 \pm 15.3 \times 10^6$ cells/rat ($P<0.01$) (Fig. 2C). Prior to administration with carrageenan, pre-treatment with IMMLG5521 at the doses of 5, 10 and 20 mg/kg reduced exudate volume to 1.11 ± 0.17 ml, 0.98 ± 0.23 ml, and 0.94 ± 0.20 ml, respectively in a dose-dependent manner. Cell migration was also reduced to $70.3 \pm 12.7 \times 10^6$ cells, $59.8 \pm 20.3 \times 10^6$ cells, and $56.1 \pm 15.0 \times 10^6$ cells, respectively. The number of polymorphonuclears was reduced to $42.1 \pm 9.2 \times 10^6$ cells, $36.2 \pm 11.3 \times 10^6$ cells and $31.5 \pm 8.3 \times 10^6$ cells respectively in a dose-dependent manner. Prior to administration with carrageenan, pre-treatment with indomethacin (2 mg/kg) reduced

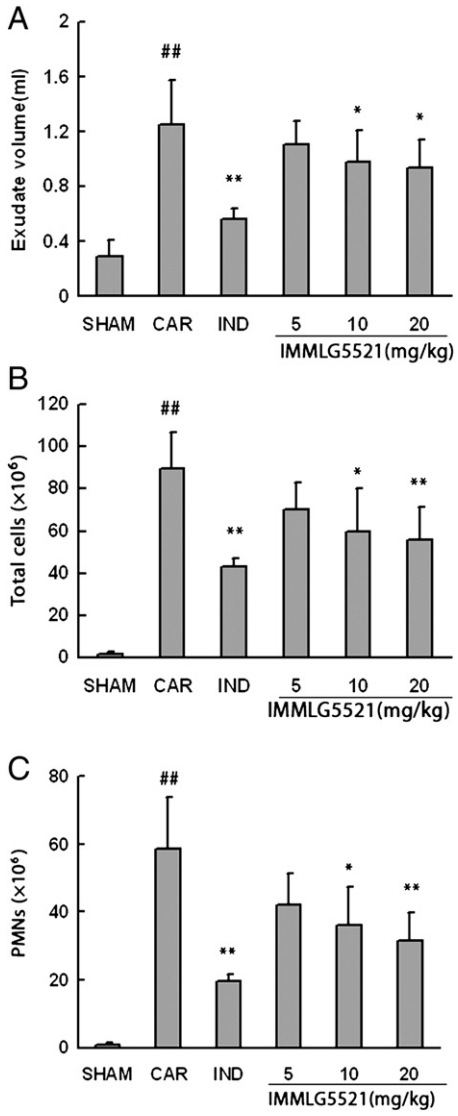

Fig. 2. Effect of IMMLG5521(5, 10 and 20 mg/kg) on carrageenan-induced inflammation. Exudate volume (A) and accumulation of leukocyte cells migration into pleural cavity of rats (B) polymorphonuclear leukocytes migration into the pleural cavity of rats (C) in pleural cavity at 4 h after carrageenan injection. Data are shown as means ± S.D. (n = 8. ##P<0.01 compared with sham-operated group; *P<0.05, **P<0.01 compared with carrageenan group).

exudate volume to 0.56 ± 0.08 ml, cell migration to 43.3 ± 3.4 × 10^6 cells, and the number of neutrophils to 19.7 ± 2.0 × 10^6 respectively.

3.2. Histological analysis of lung tissues

Comparing with the micrographs in rats treated with saline (Fig. 3A), the pathological changes of lungs in the rats treated with carrageenan showed inflammatory cells infiltration, local edema and slight congestion (Fig. 3B), while rats pre-treated with indomethacin (2 mg/kg) showed reduced changes (Fig. 3C). And rats pre-treated with the compound (20 mg/kg) showed reduced changes (Fig. 3D).

3.3. Myeloperoxidase activity in the pleural exudates

MPO activity in the supernatant of centrifuged exudates increased significantly from 19.5 ± 8.7 to 233.9 ± 76.2 U/l after carrageenan injection. (P<0.01) (Fig. 4). However, in 5, 10 and 20 mg/kg IMMLG5521-treated groups, MPO activity levels were also reduced to 123.2 ± 48.2, 110.1 ± 30.4 and 84.11 ± 27.8 U/l respectively.

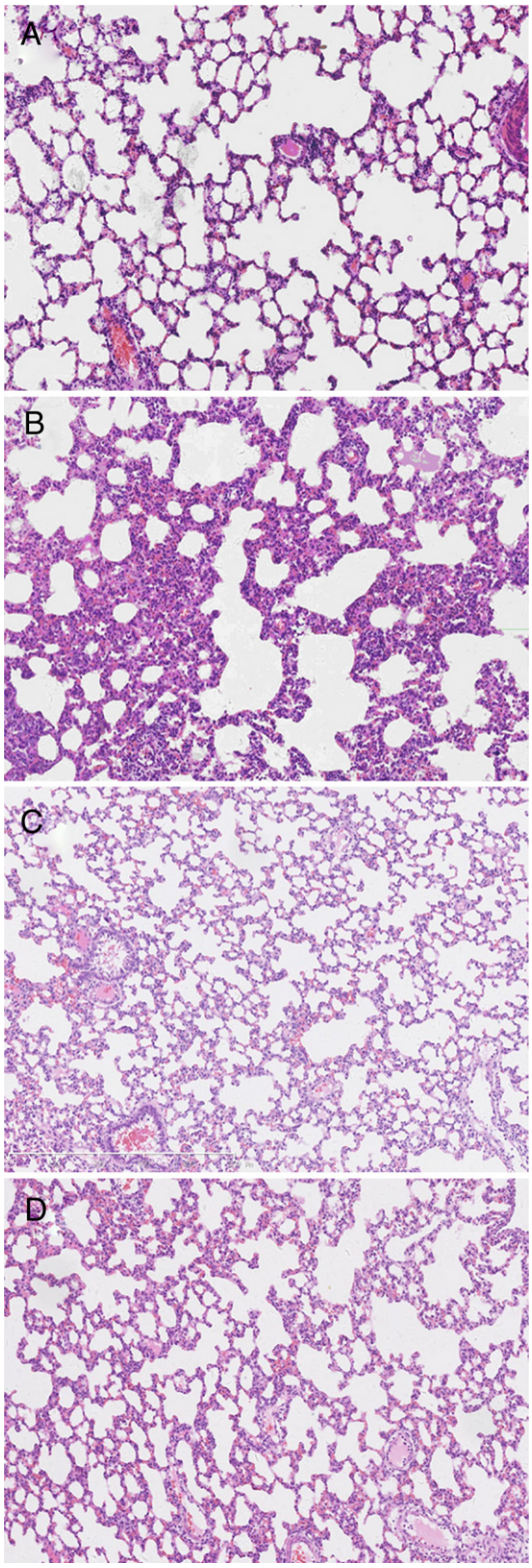

Fig. 3. Effect of the compound on lung injury(HE × 100). Normal pulmonary tissue in the rats treated with saline (A). Lung inflammation in the rats treated with carrageenan (B). Relieved lung inflammation in the rats treated with carrageenan and indomethacin (2 mg/kg) (C).Relieved lung inflammation in the rats treated with carrageenan and compound (20 mg/kg) (D).

3.4. TNF-α and IL-1β levels

The significant increase in TNF-α (Fig. 5A) and IL-1β (Fig. 5B) production was found in the supernatant of centrifuged exudates ($P<0.01$) (Fig. 5A and B respectively). TNF-α and IL-1β levels in pleural exudates were significantly reduced in a dose-dependent manner in IMMLG5521-treated rats ($P<0.05$, $P<0.01$).

3.5. MIP-2 and IL-8 levels

The significant increase in MIP-2 (Fig. 6A) and IL-8 (Fig. 6B) production was found in the supernatant of centrifuged exudates ($P<0.01$). MIP-2 and IL-8 levels in pleural exudates were significantly reduced in a dose-dependent manner in IMMLG5521-treated rats ($P<0.05$, $P<0.01$).

3.6. Effect of IMMLG5521 on NF-κB signal pathway

To examine the molecular mechanisms responsible for mediating the anti-inflammatory effects of IMMLG5521, we measured the changes of NFκB in cytosol and nuclear and IκB-α in the cytosol by western blot analysis. The results showed that that treatment with IMMLG5521 inhibited the degradation of IκB-α in the cytosol, decreased the level P65 in nuclear and increase the level of P65 in the cytosol, which indicated that IMMLG5521 down-regulated P65 activation by inhibiting degradation of IκB-α in the cytosol (Fig. 7).

4. Discussion

In the present study, we showed that IMMLG5521, a novel coumarin derivative, attenuated inflammatory response to carrageenan-induced pleurisy in rats. Further studies showed that the compound may exert its anti-inflammation effect, which might be related with decreasing the release of inflammatory factors and inhibiting NF-κB signal pathway. These findings indicated that IMMLG5521 may be useful for mitigating inflammation.

Carrageenan-induced pleurisy, a well-established inflammation model, is commonly used to investigate pathophysiology of acute inflammation, which allowed quantification and correlation of both exudate and cellular migration with changes of other inflammatory parameters (Saleh et al., 1999). Recruitment and activation of PMNs reflects a primary immunological response to invading pathogens and has also emerged as a hallmark of vascular inflammation. Neutrophils adhering to the endothelial layer might become activated by mediators locally released and increased the vascular permeability (Severino et al., 2009). Our data supported the previous study that the injection of carrageenan into pleural cavity of rats elicited an acute inflammatory response, characterized by infiltration and accumulation of fluid (edema) containing a large amount of PMNs. Treatment of the animals with IMMLG5521 (5, 10 and 20 mg/kg) attenuated the number of total leukocytes, polymorphonuclear cells and pleural volume of exudate after carrageenan challenge. The heme protein MPO is one of the principal enzymes released upon PMN activation and used as a marker enzyme for measuring PMN accumulation in tissue samples. The enzyme not only generates cytotoxic oxidants but also impacts deleteriously on nitric oxide-dependent signaling cascades within the vasculature. The results showed that IMMLG5521 inhibited MPO activity in the pleural exudates. The pathological

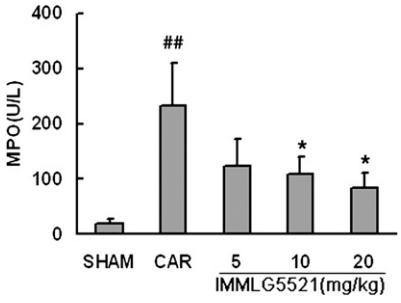

Fig. 4. Effect of IMMLG5521 (5, 10 and 20 mg/kg) on myeloperoxidase activity in the pleural exudates from the rats model of pleurisy. Data are shown as means ± S.D. (n = 6. ##P<0.01 compared with sham-operated group;*P<0.05 compared with carrageenan group).

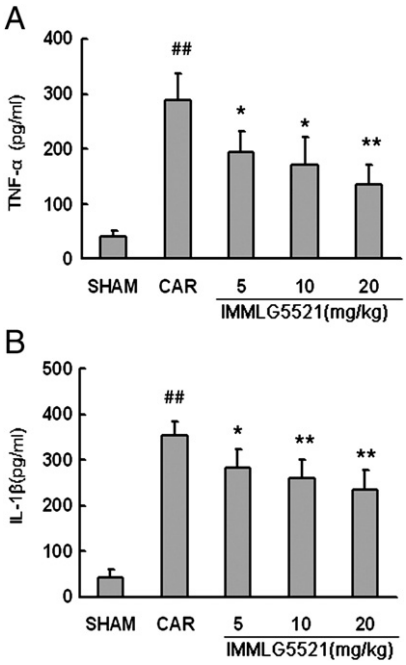

Fig. 5. Effect of IMMLG5521 (5, 10 and 20 mg/kg) on levels of TNF-α (A) and IL-1β (B) level in the pleural exudates from the rats model of pleurisy. Data are shown as means ± S.D. (n = 6. ##P<0.01 compared with sham-operated group; *P<0.05, **P<0.01 compared with carrageenan group).

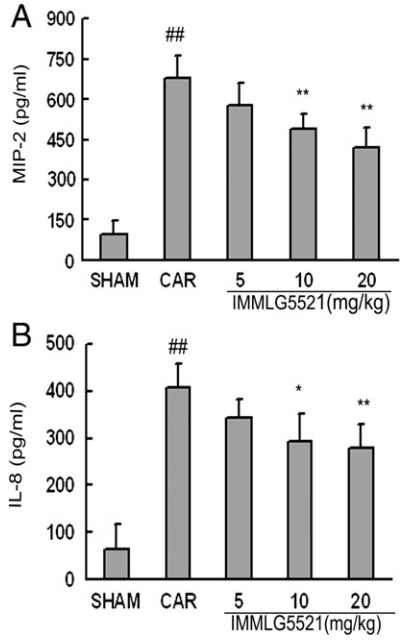

Fig. 6. Effect of IMMLG5521 (5, 10 and 20 mg/kg) on levels of MIP-2 (A) and IL-8(B) level in the pleural exudates from the rats model of pleurisy. Data are shown as means ± S.D. (n = 6. ##P<0.01 compared with sham-operated group; *P<0.05, **P<0.01 compared with carrageenan group).

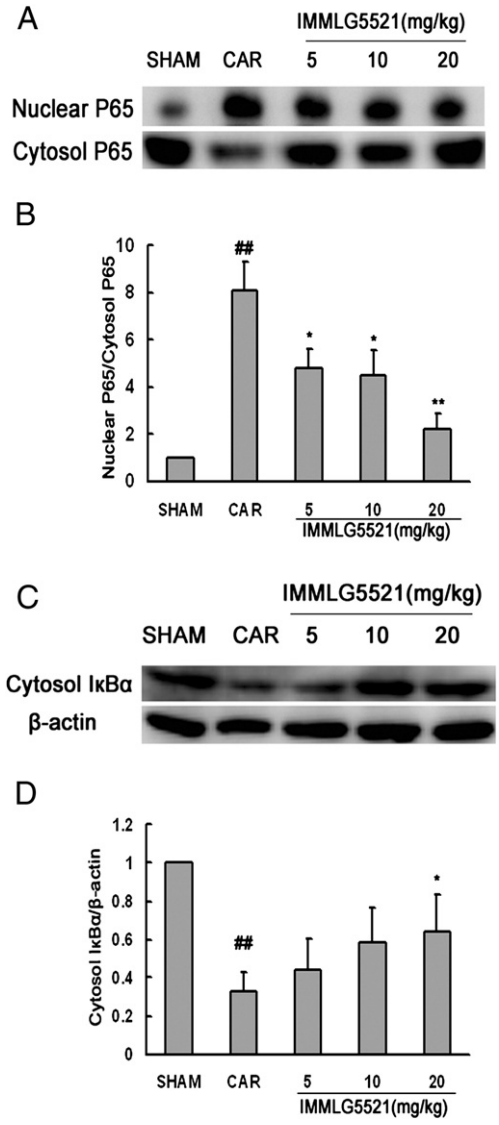

Fig. 7. Effect of compound IMMLG5521 on NF-κB activation and IκB-α degradation in inflammatory cells of pleural exudates (A, C). Quantitative analysis of p65 translocation and IκB-α degradation (B, D) (n = 3. $^{\#\#}P<0.01$ compared with sham-operated group; $^{*}P<0.05$, $^{**}P<0.01$ compared with carrageenan group).

changes of lungs in the rats treated with carrageenan showed inflammatory cells infiltration, local edema and slight congestion, while rats pre-treated with compound showed reduced changes, indicating that IMMLG5521 could attenuate the histological pattern of lung injury by inhibiting the influx of leukocytes into the lung tissue.

Activation of cytokines and mediators is the key procedure of inflammatory reaction and leads consequent inflammatory impairment and restoration (Zhao et al., 2007). Pro-inflammatory cytokines TNF-α and IL-1β are released in the pleural exudates induced by carrageenan in rats (Utsunomiya et al., 1991). These cytokines can cause chemotaxis to attract granulocytes and monocytes, migrating leukocytes produce, in turn, further cytokines, such as TNF-α and IL-1β, and other pro-inflammatory mediators (Utsunomiya et al., 1998). These cytokines have been proposed as crucial mediators for the development of carrageenan-induced pleurisy and for the accumulation of leukocytes in the inflammatory site. There is good evidence that TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (Saklatvala, 1986; Henderson and Pettipher, 1989; Piguet et al., 1992; Wooley et al., 1993). Macrophage inflammatory protein 2 (MIP-2) is a chemokine that attracts neutrophils. IL-8 is known as neutrophil chemotactic factor which promotes neutrophil chemotaxis and degranulation. CXC chemokines MIP-2 and IL-8 are important for neutrophil recruitment. This study demonstrated that IMMLG5521 significantly attenuated the production of TNF-α, IL-1β, MIP-2 and IL-8 in pleural exudates of carrageenan-injected rats.

The secretion of pro-inflammatory cytokines is under the control of the transcription factors of the nuclear factor kappa B family. It is well established that the nuclear accumulation of NF-κB relies in large part upon IκB kinase-dependent phosphorylation and subsequent degradation of the cytosolic inhibitor, IκB-α. Signaling stimulated by lipopolysaccharide and cytokines triggers the phosphorylation and degradation of IκB-α resulting in the dissociation of NF-κB from IκB-α. This phenomenon allows NF-κB dimers to migrate to the nucleus where it binds to promoters of NF-κB regulated genes and initiates gene transcription (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995). The transcription factor nuclear factor kappa B (NF-κB) has been shown to be a major regulator which can regulate many functionally diverse pro-inflammatory mediators. NF-κB is a general term used to describe a number of dimeric combinations of members of the Rel family of gene regulatory proteins that possess transcriptional activating properties (Ghosh et al., 1998). Some studies have reported that intrapleural injection of carrageenan caused activation of NF-κB which plays an important role in the development of inflammatory response (D'Acquisto et al., 1999; Ianaro et al., 2001). The result demonstrated that the anti-inflammatory activity of IMMLG5521 led to the inhibition of NF-κB activation by reducing the phosphorylation of IκB-α in the inflammatory cells and by inhibiting the degradation of IκB-α in the cytoplasm. As a final result, the abnormal translocation of NF-κB into the nucleus was stopped by IMMLG5521. The exact mechanisms by which IMMLG5521 suppresses NF-κB activation in inflammation remain to be further elucidated.

In conclusion, our current study demonstrated that IMMLG5521, a novel coumarin derivative, reduced the inflammatory reactions induced by carrageenan in rats. Treatment of the animals with IMMLG5521 (5, 10 and 20 mg/kg) attenuated the total leukocytes, number of polymorphonuclear cells and pleural volume of exudate after carrageenan challenge. Such results revealed that the IMMLG5521 displayed significantly anti-inflammatory activity, via inhibiting vascular permeability and leukocyte transmigration, which might be related with the reduction of release of inflammatory factors, another part, may be related to an inhibition of NF-κB activation. Compound IMMLG5521 showed significant anti-inflammatory effect on carrageenan-induced pleurisy in rats. However, further studies are required to elucidate its underlying action mechanism.

Acknowledgment

The authors would like to thank professor Da-yong Cai for kindly providing assistance in pathological study. This work was supported in part by grants from the Nature Science Foundation of China (No. 90713045, No. 30801527, No. 30973887, Key Program No. U0832008), the National 863 Program of China (Grant No. 2006AA020501) and National Key Sci-Tech Major Special Item (No. 2009ZX0 9301-003).

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