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# Ceftiofur impairs pro-inflammatory cytokine secretion through the inhibition of the activation of NF-κB and MAPK

Xinxin Ci [a,1], Yu Song [a,1], Fanqin Zeng [a,1], Xuemei Zhang [a], Hongyu Li [a], Xinrui Wang [a], Junqing Cui [b,*], Xuming Deng [a,*]

[a] Department of Veterinary Pharmacology, College of Animal Science and Veterinary Medicine, Jilin University, Xi'an Road 5333, Changchun, Jilin 130062, People's Republic of China
[b] Wyeth Research, 200 Cambridge Park Drive, Cambridge, MA 02140, USA

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ABSTRACT

Ceftiofur is a new broad-spectrum, third-generation cephalosporin antibiotic for veterinary use. Immunopharmacological studies can provide new information on the immunomodulatory activities of some drugs, including their effect on cytokine productions. For this reason, we investigated the effect of ceftiofur on cytokine productions in vitro. We found that ceftiofur can downregulate tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), but did not affect interleukin-10 (IL-10) production. We further investigated signal transduction mechanisms to determine how ceftiofur affects. RAW 264.7 cells were pretreated with 1, 5, or 10 mg/L of ceftiofur 1 h prior to treatment with 1 mg/L of LPS. Thirty minutes later, cells were harvested and mitogen activated protein kinases (MAPKs) activation was measured by Western blot. Alternatively, cells were fixed and nuclear factor-κB (NF-κB) activation was measured using immunocytochemical analysis. Signal transduction studies showed that ceftiofur significantly inhibited extracellular signal-regulated kinase (ERK), p38, and c-jun $NH_2$-terminal kinase (JNK) phosphorylation protein expression. Ceftiofur also inhibited p65-NF-κB translocation into the nucleus. Therefore, ceftiofur may inhibit LPS-induced production of inflammatory cytokines by blocking NF-κB and MAPKs signaling in RAW264.7 cells.



Infections with Gram-negative bacteria result in significant damage to the body including sepsis syndrome, which is characterized by hypotension, tachycardia, tachypnea, disseminated intravascular coagulation, and multiple organ system failure [1]. In the last several years, many scientists have attempted to define the pathogenic mechanisms responsible for the inflammatory responses that result in sepsis syndrome. LPS activates the Toll-like receptor-4 (TLR4) complex on host cells, such as monocytes and macrophages, and initiates the systemic inflammatory response that accompanies sepsis. This systemic response is characterized by the release of pro-inflammatory cytokines and other inflammatory mediators, including TNF-α, IL-1, IL-6, IL-12, interferon (IFN) and nitric oxide (NO) [2,3]. The increased production of these inflammatory mediators may result in severe tissue damage and septic shock. Currently, treatment of sepsis relies largely on intravenous administration of antibiotics that can downregulate pro-inflammatory cytokines. However, under some circumstances, these conventional therapies may promote the additional release of LPS from the cell envelope of killed bacteria, exacerbating sepsis itself [4]. For this reason, it is necessary to find more effective drugs that have anti-inflammatory effects. Based on recent studies [5,6], some antibacterial agents that downregulate pro-inflammatory cytokines and (or) upregulate anti-inflammatory cytokines, may have therapeutic effects on septic shock.

Ceftiofur is a new broad-spectrum, third-generation cephalosporin antibiotic for veterinary use. Ceftiofur inhibits bacterial cell wall synthesis by interfering with enzymes essential for peptidoglycan synthesis, which results in lysis of the bacterial cell and accounts for the bactericidal nature of this antibiotic [7]. Consequently, ceftiofur should be effective against a wide range of contagious and environmental mastitis pathogens. It has been widely used to treat a broad array of infectious diseases caused by Gram-positive and Gram-negative bacteria, such as pneumonia, peritonitis, and mastitis in dairy cattle [8]. Among third-generation cephalosporins, cefodizime was shown to modulate the release of inflammatory cytokines [9]. Importantly, ceftiofur has a similar structural formula to cefodizime, though ceftiofur's effect on septic shock has not yet been reported. Therefore, we investigated

* Corresponding authors. Fax: +1 617 665 5584 (J. Cui), +86 431 87836160 (X. Deng).
*E-mail addresses:* jcui@wyeth.com (J. Cui), xumingdeng@jluhp.edu.cn (X. Deng).
[1] These authors contributed equally to this work.

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whether ceftiofur has any anti-inflammatory effects. In this study, we demonstrate that ceftiofur downregulates pro-inflammatory cytokines in vitro.

The nuclear factor-κB (NF-κB) is essential for host defense and inflammatory responses to microbial and viral infections [10]. In response to extracellular stimuli such as LPS, TNF-α or other inflammatory mediators, the transcription factor NF-κB is often activated and subsequently facilitates the transcription of a number of genes involved in inflammation [11]. The other major extracellular signal transduction pathway stimulated by inflammatory mediators is MAPKs pathway [12]. MAPKs are a family of proteins, including ERK, p38, and JNK [13]. LPS, a key mediator in the inflammatory response, can induce activation of these MAPKs proteins in macrophages and other cell types [12,14,15]. NF-κB and MAPKs are therefore known as important targets for anti-inflammatory molecules. Therefore, we investigated whether ceftiofur can activate the NF-κB and MAPKs pathways.

## Materials and methods

*Reagents.* Ceftiofur, dimethyl sulfoxide (DMSO), LPS (*Escherichia* coli 055:B5), and 3-(4,5-dimethylthiazol-2-y1)-2,5-dipheny-ltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). Rabbit anti-NF-κB/p65 polyclonal antibody, rabbit anti-JNK polyclonal antibody, rabbit anti-ERK polyclonal antibody, rabbit anti-p38 polyclonal antibody, mouse anti-phospho-JNK monoclonal antibody, mouse anti-phospho-ERK monoclonal antibody, and mouse anti-phospho-p38 monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Cy3-conjugated sheep antirabbit IgG, peroxidase-conjugated Affinipure goat anti-mouse IgG (H + L) and peroxidase-conjugated Affinipure goat antirabbit IgG (H + L) were purchased from PTG (Chicago, IL, USA).

*Cell culture.* The RAW264.7 mouse macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). Cells were cultured in DMEM supplemented with 3 mM glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and 10% heat-inactivated fetal bovine serum. The cells were maintained at 37 °C in a humidified incubator containing 5% $CO_2$. In all experiments, cells were allowed to acclimate for 24 h before any treatments. Ceftiofur was always added 1 h prior to LPS treatment.

*MTT assay for cell viability.* To measure cell viability, the MTT assay was performed. RAW 264.7 cells were mechanically scraped, seeded in 96-well plates at $4 \times 10^5$ cells/mL, and incubated in a 37 °C, 5% $CO_2$ incubator overnight. After 24 h, the cells were treated with 50 μL different concentrations of ceftiofur (0–10 mg/L) for 2 h, followed by stimulation with 50 μL of LPS for 18 h. Subsequently, 20 μL of 5 mg/mL MTT in FBS-free medium was added to each well and incubated for an additional 4 h. Cell-free supernatants were then removed and resolved with 150 μL/well DMSO. The optical density was measured at 570 nm on a microplate reader.

*Monitor cytokine in vitro.* To investigate the effect of ceftiofur on cytokine responses from LPS-treated cells, RAW 264.7 cells ($4 \times 10^5$) were seeded into 24-well plates, pretreated with 1, 5 or 10 mg/L of ceftiofur for 1 h prior to treatment with 1 mg/L of LPS for 12 h. Cell-free supernatants were collected and stored at −20 °C until assayed for cytokine levels. The concentrations of TNF-α, IL-1β, IL-6, and IL-10 in the cell supernatants, were determined using an ELISA kit. Concentrations were determined for six wells of each sample.

*Western blot analysis.* RAW 264.7 cells ($4 \times 10^5$) were cultured in 6-well plates for 24 h, pretreated with 1, 5 or 10 mg/L of ceftiofur for 1 h prior to treatment with 1 mg/L of LPS for 30 min. The cells were collected on ice, washed twice with ice-cold PBS and suspended in 40 μL of lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6% NP-40, 1 mM $Na_3VO_4$, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM *p*-nitrophenyl phosphate and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany)). After the lysates were incubated on ice for 30 min, they were centrifuged (12,000*g* at 4 °C) for 5 min to obtain the cytosolic fraction. The protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany) before storage at −70 °C. Samples were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were then washed in Tris–Tween-buffered saline [TTBS, 20 mM Tris·HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05% (vol/vol) Tween 20], blocked overnight with 5% (wt/vol) nonfat dry milk, and probed according to the method described by Towbin et al. [16] with monoclonal phospho-specific antibodies to p46–p54 JNK, p42/p44 ERK, and $p38^{mapk}$ in 5% (wt/vol) bovine serum albumin (BSA) dissolved in TTBS. With the use of peroxidase-conjugated secondary anti-mouse antibody, bound antibodies were detected using ECL plus (GE Healthcare). To confirm equal loading of proteins between samples, the membranes were probed with rabbit polyclonal p54 JNK, p42 ERK, and $p38^{mapk}$ antibodies.

*Immunocytochemical analysis.* RAW 264.7 cells ($4 \times 10^5$) cultured on glass coverslips were plated into 24-well plates for 24 h, pretreated with 1, 5, or 10 mg/L of ceftiofur 1 h prior to treatment with 1 mg/L of LPS for 1 h. Glass coverslips were washed with 0.01 M PBS and fixed in 4% formaldehyde for 30 min at room temperature. Detergent extraction with 3% Triton X-100 was performed for 10 min at room temperature. Coverslips were then saturated with PBS containing 5% BSA for 30 min at room temperature and processed for immunofluorescence with rabbit anti-NF-κB/p65 polyclonal antibody followed by Cy3-conjugated sheep antirabbit IgG. Finally, coverslips were mounted on slides and fluorescence signals were analyzed by Fluoview microscopy (OLYMPUS, Japan).

*Statistical analysis.* All values were expressed as means ± the standard error of the mean (SEM). Differences between mean values of normally distributed data were assessed by the one-way ANOVA (Dunnett's *t*-test) and the Student's *t*-test. Statistical difference was accepted at $P < 0.05$.

## Results

### *The effect of ceftiofur on macrophage toxicity*

Ceftiofur did not display any cellular toxicity against RAW264.7 cells over 24 h, as determined by the MTT assay (Fig. 1).

### *The effect of ceftiofur on LPS-induced cytokine production in vitro*

TNF-α, IL-1β, IL-6, and IL-10 concentrations in the culture supernatants of RAW 264.7 cells were measured by sandwich ELISA (Fig. 2). RAW 264.7 cells treated with LPS produced significant amounts of all cytokines examined. The concentration of TNF-α was up to 18 ng/mL after LPS stimulation. However, the concentration of TNF-α in the supernatant of cells pretreated with 1, 5, or 10 mg/L of ceftiofur was significantly decreased compared to the LPS control group ($^{*}P < 0.05$, $^{**}P < 0.01$). IL-6 levels also decreased significantly after pretreatment with 1, 5, or 10 mg/L ceftiofur compared to the LPS group ($^{*}P < 0.05$, $^{**}P < 0.01$). IL-1β levels decreased significantly at 10 mg/L ($^{**}P < 0.01$) ceftiofur pretreatment, although the concentration

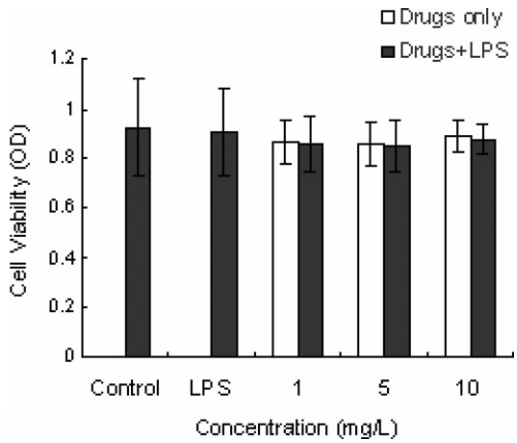


**Fig. 1.** Effect of ceftiofur on the viability of RAW264.7 cells. Cells were cultured with ceftiofur (0–10 g/mL) in the absence or presence of 1 g/mL LPS for 24 h. Data are presented as means ± SEM of three independent experiments.

was low compared to that of TNF-α. The concentration of IL-10 was slightly increased in groups treated with ceftiofur and showed no significant change compared to the LPS control group ($P > 0.05$).

*The effects of ceftiofur on LPS-induced MAPK pathway activation*

To fully understand the mechanism by which inhibits LPS-induced production of inflammatory cytokines, we next investigated the possible connection between increasing concentrations of ceftiofur and MAPKs pathways activation (Fig. 3). After cells were stimulated with LPS, the levels of phosphorylation MAPKs were subsequently measured by Western blot analysis using three different phospho-specific antibodies. The phosphorylation levels of the MAPKs isoforms decreased dramatically in ceftiofur-treated cells compared with the LPS-treated control cells (1 mg/L). However, there was no change in the no phosphorylation levels of the MAPKs isoforms between the different groups. Total protein levels of MAPKs were used as a control for possible fluctuations in MAPKs levels.

*The effects of ceftiofur on the activation of NF-κB*

We evaluated the effect of ceftiofur on LPS-induced activation of the NF-κB pathway and found that p65 was distributed in the cytoplasmic compartment before LPS stimulation. Treatment with LPS resulted in the accumulation of p65 in the nucleus. Thirty minutes after stimulation with LPS (1 mg/L), the majority of intracellular p65 translocated from the cytoplasm to the nucleus, as shown by strong p65 staining in the nuclear (Fig. 4). However, in LPS-treated samples that were pre-treated with ceftiofur, nuclear translocation of p65 was strongly inhibited, in a dose-dependent manner (Fig. 4).

## Discussion

When mononuclear phagocytes recognize LPS via cell surface receptors, they release numerous pro-inflammatory cytokines [17]. Excessive production of pro-inflammatory cytokines will result in a systemic inflammatory response syndrome typified by septic shock and the complications that are normally associated with it. It would be desirable to be able to selectively regulate cytokine production in the treatment of inflammatory diseases [18–21]. Therefore, any intervention that inhibits the release of pro-inflammatory cytokines or upregulates anti-inflammatory cytokines is believed to benefit patients experiencing septic shock. Among the inflammatory cytokines, TNF-α plays a key role in regulating inflammation, mostly through the induction of other inflammatory cytokines including IL-1 (IL-1α and IL-1β), IL-6, IL-8, macrophage inflammatory protein 2, granulocyte-macrophage colony-stimulating factor and adhesion molecules [22,23]. This present study demonstrates that ceftiofur inhibits production of TNF-α, IL-6, and IL-1β in LPS-stimulated RAW264.7 cells in a dose-dependent manner. LPS induces TNF-α and other inflammatory gene expression by activating MAPKs and the transcription factors NF-κB and C/EBP in macrophages [24,25]. To further characterize the nature of the inhibitory effect of ceftiofur on cytokine production, we examined the effects of ceftiofur on the activation of the MAPKs p38, ERK and JNK, which are known to be involved in the regulation of these cytokines. We also examined the effects of ceftiofur on the activation

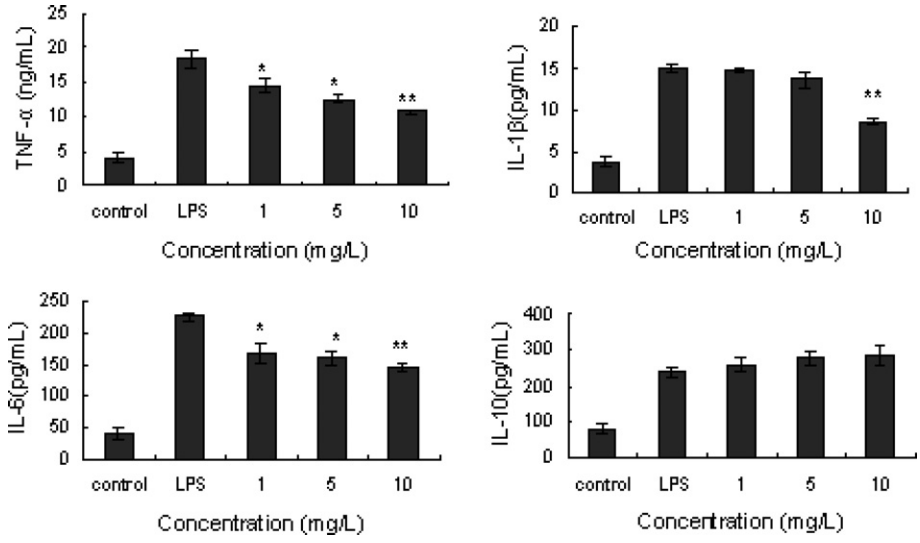


**Fig. 2.** Effect of different concentrations of ceftiofur on the secretion of TNF-α, IL-1β, IL-6 and IL-10 in vitro. The cells were treated with LPS alone or LPS plus different concentrations (1, 5 or 10 mg/L) of ceftiofur for 12 h. Control values were obtained in the absence of LPS or ceftiofur. The values represent means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

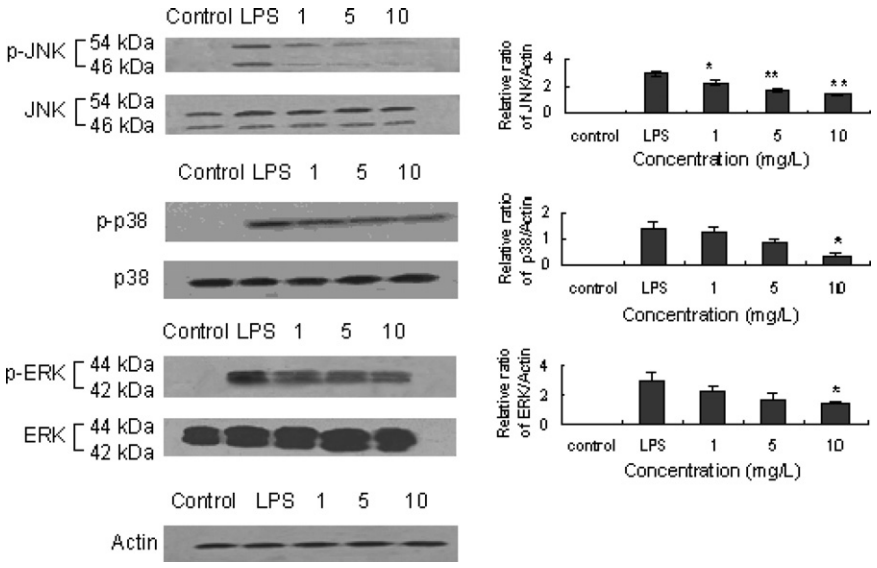


**Fig. 3.** Effect of ceftiofur on LPS-induced phosphorylation of MAPK. The cells were stimulated with or without LPS (1 mg/L) for 30 min. Protein samples were analyzed by Western blot with phospho-specific antibodies. The total MAPK levels were used as an internal control. Shown in the right panel are means ± SEM of three independent experiments. A representative Western blot is shown in the left panel.

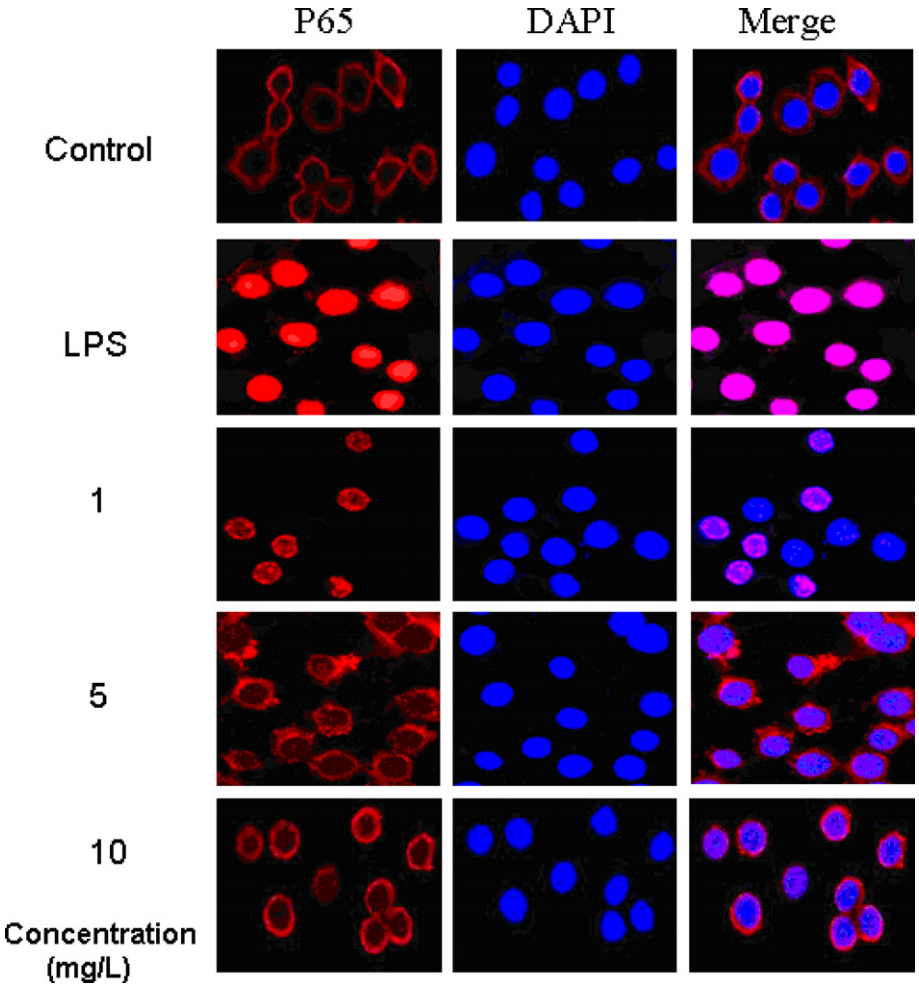


**Fig. 4.** Effect of ceftiofur treatment on nuclear translocation of NF-κB induced by LPS. Immunocytochemistry techniques were employed. Cells were cultured for 1 h with LPS (1 mg/L), fixed, permeabilized, and incubated with rabbit anti-p65 antibody followed by Cy3-conjugated antirabbit Ig (red). The nuclei of the corresponding cells were visualized by DAPI staining. Magnification for images was 600×. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

of the transcription factor NF-κB, which regulates the expression of many immune and inflammatory genes.

Several reports have shown that the LPS signaling cascade leading to TNF-α production in macrophages and monocytes is dependent on the activation of the members of the MAPKs family: p38, ERK1/2 and JNK [24,26,27]. Several natural products have been shown to inhibit the expression of these genes by modulating the phosphorylation of MAPKs pathways. For example, luteolin inhibits LPS-induced TNF-α production in RAW264.7 cells by simultaneous inhibition of the ERK1/2 and p38 pathways [28]. Therefore, it has been suggested that ceftiofur-mediated downregulation of pro-inflammatory cytokines occurs through the inhibition of the activation of MAPKs signaling. As shown by our experiments, LPS induces rapid phosphorylation of ERK1/2, JNK and p38 kinase in RAW264.7 cells, which ceftiofur treatment impairs phosphorylation of these molecules in a dose-dependent manner.

Recent studies have shown that NF-κB is essential for host defense and inflammatory responses to microbial and viral infections. The expression of many inflammation-related genes is regulated through the NF-κB signaling pathway [29]. In our studies, we found that LPS-induced NF-κB p65 translocation from the cytoplasm to the nucleus was strongly inhibited by ceftiofur treatment (Fig. 4). Our results suggest that ceftiofur suppresses LPS-induced pro-inflammatory cytokine production by inhibiting the activation of both MAPKs and the transcription factor NF-κB in RAW264.7 cells.

Future studies should focus on the basic mechanisms governing ceftiofur inhibition of pro-inflammatory cytokine production. In addition, future studies should also address the on clinical relevance of our studies. It is not clear at present how ceftiofur inhibits the NF-κB and MAPKs signaling in stimulated cells, and events upstream of the TLR level should be investigated. From a clinical standpoint, the anti-inflammatory effects of ceftiofur could be of significant importance when these effects are linked to its antimicrobial properties. The effects of ceftiofur on lung infection, inflammation, and tissue injury should be further studied in animal models.

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