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Article

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Biapenem Attenuates LPS-Induced Severe Pneumonia via Modulation of the
TLR4/NF- κ B/PI3K-AKT Signaling Axis

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Abstract

Background: Severe pneumonia is a leading cause of ICU admission and mortality worldwide. Uncontrolled host inflammatory responses, rather than pathogens, are the core mechanism driving ARDS and multiple organ failure. Current therapies lack agents combining potent antibacterial activity with safe anti-inflammatory effects. Biapenem, a carbapenem with excellent pulmonary penetration, has shown immunomodulatory activity, but its direct effects on the TLR4/NF- κ B/PI3K-AKT axis and concentration-dependent characteristics remain unclear.

Methods: Network pharmacology and molecular docking identified key targets of biapenem in severe pneumonia. LPS-stimulated A549 cells were treated with low, medium, and high biapenem concentrations; dexamethasone served as positive control. Western blot detected TLR4, AKT, p-AKT, P65, and p-P65; q-PCR measured TLR4, NF- κ B p65, AKT1, IL-1 β , IL-6, and TNF- α mRNA; ELISA quantified the three inflammatory cytokines in supernatant.

Results: Network pharmacology identified 78 intersecting targets between biapenem and severe pneumonia, with key targets including AKT1, EGFR, PPARG, JAK2, KIT, PTPN11, ACE, PLG, and SYK. Molecular docking confirmed stable biapenem-AKT1 binding. LPS stimulation elevated TLR4, p-AKT, p-AKT/AKT ratio, and p-P65, while total AKT remained unchanged. Biapenem suppressed p-P65 phosphorylation, dually blocked NF- κ B and PI3K/AKT pathways, and downregulated TLR4, thereby extinguishing inflammatory signaling. q-PCR, Western blot, and ELISA results were highly consistent.

34 Conclusion: Biapenem exerts anti-inflammatory effects in LPS-stimulated A549
35 alveolar epithelial cells by inhibiting the TLR4/NF- κ B/PI3K-AKT signaling axis. This study
36 establishes the molecular basis for the dual functionality of biapenem, encompassing both
37 anti-infective and anti-inflammatory properties, thereby providing a novel therapeutic
38 strategy for inflammatory diseases such as severe pneumonia.

39
40 Keywords: biapenem, severe pneumonia, TLR4/NF- κ B, PI3K/AKT, inflammation
41

Introduction

Severe pneumonia is a leading cause of hospitalization and mortality worldwide (Gazivoda et al., 2021, Chen et al., 2023). Despite advances in antimicrobial therapy and intensive care, mortality remains high. A systematic review of 48,707 ICU patients found SCAP mortality of 37.1% in low- and middle-income countries, rising to 59.3% in mechanically ventilated patients, versus 16–26% in high-income countries (Jiang et al., 2020a). Hypertension, COPD, and diabetes are the most common comorbidities (Chetboun et al., 2021).

The high mortality of severe pneumonia is fundamentally driven by uncontrolled host inflammatory responses rather than pathogens themselves (Guo et al., 2025). Even with adequate anti-infective therapy, excessive pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 can trigger a "cytokine storm," leading to diffuse alveolar damage, ARDS, and multiple organ failure (Mou et al., 2026). Current treatment mainly relies on early broad-spectrum antibiotics and empirical anti-infective coverage (Espejo-Castellanos et al., 2026), yet antimicrobial therapy alone cannot effectively contain excessive inflammatory injury. Although glucocorticoids such as dexamethasone have broad-spectrum anti-inflammatory effects, their use in severe pneumonia remains controversial due to concerns including secondary infection risk from immunosuppression, hyperglycemia, myopathy, and long-term adverse effects (Van Almsick et al., 2022, Noreen et al., 2021). Therefore, therapeutic strategies combining potent bactericidal activity with a safe anti-inflammatory profile are urgently needed.

Gram-negative bacteria are major pathogens in severe pneumonia, and LPS, their outer membrane core component, is the classic pathogen-associated molecular pattern triggering pulmonary inflammation. TLR4 is the key pattern recognition receptor for LPS (Manivannan et al., 2025). This releases NF- κ B dimers (mainly p65/p50) for nuclear translocation, initiating pro-inflammatory gene transcription (Liu et al., 2024, Choubey and Gutterman, 1996). Phosphorylated p65 (p-P65) is a critical marker of NF- κ B activity, directly regulating IL-1 β , IL-6, and TNF- α expression (Okan et al., 2023).

Furthermore, the PI3K/AKT signaling pathway also plays a pivotal role in inflammatory regulation. AKT can directly phosphorylate IKK α to facilitate NF- κ B activation, and can also activate mTORC1 to upregulate HIF-1 α , enhancing transcriptional capacity of inflammatory cytokines such as IL-1 β (Chen et al., 2025, Babaev et al., 2016). Moreover, TNF- α induced by NF- κ B activation can in turn activate PI3K/AKT through its receptors, constituting a vicious cycle of mutual reinforcement (Li et al., 2017). Studies have shown that both PI3K/AKT and NF- κ B pathways are activated in immune cells stimulated by LPS or carbapenem-resistant *Klebsiella pneumoniae*, and simultaneous targeting of both pathways significantly enhances anti-inflammatory efficacy (Jiang et al., 2020b). Therefore, inhibiting a single inflammatory pathway alone is often insufficient, and simultaneous targeting of both PI3K/AKT and NF- κ B axes may confer synergistic anti-inflammatory advantages.

Carbonem antibiotics have the broadest antibacterial spectrum and strongest activity among β -lactams, serving as core agents for severe pneumonia, especially drug-resistant Gram-negative infections. However, accumulating evidence shows carbapenems

possess immunomodulatory activities independent of bactericidal effects (Lee et al., 2020). In a *Klebsiella pneumoniae* pneumonia mouse model, doripenem, meropenem, and imipenem all reduced bacterial burden, but with significant differences in inflammatory modulation. This suggests anti-inflammatory effects beyond bactericidal activity vary among carbapenems, potentially due to differences in penicillin-binding protein affinity and endotoxin release. Biapenem, a key carbapenem, has pharmacokinetic advantages including high stability against renal dehydropeptidase-I and excellent pulmonary penetration (Fukasawa et al., 1992). Lee et al. demonstrated biapenem significantly attenuated PM2.5-induced lung injury by downregulating TLR4 and MyD88 and modulating the mTOR–autophagy pathway, providing first evidence of biapenem's anti-inflammatory effects in pulmonary inflammation (Lee et al., 2020).

Whether biapenem directly intervenes in the LPS-triggered TLR4/NF- κ B/PI3K-AKT axis and whether its anti-inflammatory effects are concentration-dependent and multi-target remain unclear. This study hypothesizes that biapenem inhibits TLR4 and downstream PI3K/AKT and NF- κ B pathways, blocking pro-inflammatory cytokine synthesis and release, thereby exerting antibacterial and anti-inflammatory effects advantageous for severe pneumonia. To test this, network pharmacology and molecular docking were used to identify key targets, and an LPS-induced A549 inflammatory model was treated with low, medium, and high biapenem concentrations, with dexamethasone as positive control. This provides evidence supporting biapenem's rational clinical use in severe inflammatory diseases such as severe pneumonia.

Methods

Identification of Therapeutic Targets for Severe Pneumonia

The PubChem database was used to obtain the SMILES notation of biapenem, which was subsequently imported into the SwissTargetPrediction database to predict the potential targets of biapenem. The GeneCards database was searched using the keyword "severe pneumonia" to retrieve associated targets.

The Venny 2.1 online tool was employed to generate a Venn diagram illustrating the overlap between biapenem targets and severe pneumonia targets, and the intersecting targets were identified as potential therapeutic targets of biapenem for severe pneumonia. The screened potential targets were imported into Cytoscape 3.8.0 software to construct a "biapenem–target–severe pneumonia" network. The intersecting targets were subsequently imported into the STRING database, with the species set to "Homo sapiens" and the minimum required interaction score set to high confidence (0.900), to construct a protein–protein interaction (PPI) network.

To further elucidate the underlying mechanisms, the intersecting target genes were imported into the DAVID 6.8 database, with the species restricted to "Homo sapiens," for Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The analytical results were organized and visualized using online visualization tools to graphically present the enrichment networks, and the results were annotated based on the KEGG database (Kanehisa et al., 2025, Kanehisa, 2019).

Verification by Molecular Docking

Molecular docking simulations were performed using Discovery Studio 2019 software. First, the "Prepare Ligands" module was employed to prepare the structure of biapenem, generating the three-dimensional conformation for subsequent docking. Simultaneously, the protein structures were preprocessed using the "Prepare Protein" module, which included the removal of crystallographic water molecules from the protein crystal structures. Ten key targets identified from the preceding network pharmacology analysis were selected as receptors for molecular docking studies, including AKT1, TGFBR1, MAP2K1, FGFR2, FGFR1, EGFR, IGF1R, MAPK10, KIT, and CREBBP. Following the preparation of both ligand and protein structures, the "CDOCKER" module was employed to perform molecular docking simulations to evaluate the binding modes and affinities between the active small molecule and the key targets.

Cell Culture and CCK-8 Cell Viability Assay

The A549 cell line (Cat. No. TCH-C116) was obtained from HyCyte. The cell line was authenticated by STR profiling and tested negative for mycoplasma contamination. Biapenem (Cat. No. B129949-10mg) and dexamethasone (Cat. No. D754934-25mg) were purchased from Aladdin. Lipopolysaccharide (LPS, Cat. No. L6529) was obtained from Sigma. DMEM/F12 medium (Cat. No. G4612-500ml), PBS, and the CCK-8 assay kit (Cat. No. G4103) were purchased from Servicebio. Fetal bovine serum (FBS, Cat. No. FSP500) was obtained from ExCell Bio.

A549 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained at 37°C in a humidified incubator

with 5% CO₂. Cells were passaged every 2–3 days. Cell viability was evaluated using the CCK-8 assay, and the following screening experiments were performed sequentially:

First, the optimal LPS concentration for model establishment was determined. A549 cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 μ L of culture medium. The plates were incubated at 37°C in a 5% CO₂ incubator for 18 h to allow normal adherent growth. Cells were then treated with LPS at concentrations of 1, 5, 10, and 20 μ g/mL for 24 h according to the experimental groups. Subsequently, 10 μ L of CCK-8 solution was added to each well, and wells containing the corresponding amount of culture medium and CCK-8 solution without cells served as blank controls. After incubation for an additional 1 h in the cell culture incubator, the absorbance was measured at 450 nm using a microplate reader, and cell viability was calculated to determine the optimal LPS concentration for inducing inflammatory injury. Under the optimal LPS induction concentration, dexamethasone at 0.1, 1, and 10 μ g/mL was added as the positive control, and the optimal dexamethasone concentration was determined using the CCK-8 assay.

Second, the safe concentration range of biapenem was screened. After cell culture, biapenem was added at final concentrations of 10, 25, 50, 100, and 200 μ g/mL for 24 h. Cell viability was similarly assessed using the CCK-8 assay to determine the concentrations of biapenem that did not significantly affect cell viability.

Western Blot

β -actin (Cat. No. 81115-1-RR) was purchased from Wuhan Sanying. Antibodies against AKT (GB15689), p-AKT (GB150002), and TLR4 (GB11519-100) were purchased

from Servicebio, while P65 (AF5006) and p-P65 (AF2006) antibodies were obtained from Affinity.

Cells were seeded into 6-well plates at a density of 1×10^5 cells per well. After 24 h of culture, cells were treated according to the experimental groups, including the blank group (A549), the model group (LPS), the experimental groups (intervened with low, medium, and high concentrations of biapenem), and the positive control group (intervened with dexamethasone). Cells were collected, and total protein was extracted. Protein concentrations were determined using the BCA assay. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 h, followed by incubation with primary antibodies against AKT (1:1000), p-AKT (1:1000), P65 (1:1000), p-P65 (1:1000), TLR4 (1:1000), and β -actin (1:50000) at 4°C for 16 h. After washing three times with TBST, the membranes were incubated with secondary antibodies at room temperature for 30 min, and protein bands were visualized using ECL chemiluminescence reagent.

qPCR

All pipette tips and EP tubes used in qPCR experiments were disposable AXGEN RNase/DNase-free consumables.

Total RNA was extracted from A549 cells using an animal tissue/cell total RNA extraction kit (TSP413, Tsingke). Reverse transcription was performed using the SynScript® III RT SuperMix for qPCR (TSK314S, Tsingke) according to the following procedure: 6 μ L of RNA template and 1 μ L of 5 $\times$ gDNA Remover Mix were added, and RNase-free water was supplemented to a total volume of 10 μ L. The mixture was

incubated at 42°C for 2 min, followed by 60°C for 5 min. After centrifugation, 1 µL of RNasin and 4 µL of 5× SynScript® III RT were added, and RNase-free water was supplemented to 20 µL. The mixture was incubated at 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min. The reverse transcription products were placed on ice or stored under refrigeration for subsequent use. The resulting cDNA was diluted 2-fold and used as the qPCR template. Amplification was performed using Tsingke ArtiCanCEO SYBR qPCR Mix with the following reaction components: ArtiCanCEO SYBR qPCR Mix 10 µL, 10 µM forward primer 0.8 µL, 10 µM reverse primer 0.8 µL, template (cDNA) 1 µL, and ddH₂O 7.4 µL.

ELISA

ELISA kits for IL-1β (Cat. No. ABE-R1797), IL-6 (Cat. No. ABE-R1823), and TNF-α (Cat. No. ABE-H2483) were purchased from Abeomics. A total of 1×10^6 cells were resuspended in 150–200 µL of PBS and lysed by repeated freeze–thaw cycles or sonication. The resulting lysates were centrifuged at $1,500 \times g$ for 10 min at 2–8°C, and the supernatants were collected for detection using the respective ELISA kits.

Statistical analysis

For Western blotting, the gel images obtained after exposure were opened using ChemiScope Capture image acquisition software, organized using Photoshop, and analyzed using ImageJ 1.45 software to quantify the grayscale values of the target bands. The grayscale values were imported into Excel to generate bar charts for densitometric analysis.

For ELISA, the optical density (OD) values of each well were measured at 450 nm using a microplate reader within 5 min after termination of the reaction. The mean OD values of the standards and sample replicates were calculated and corrected by subtracting the OD values of the blank wells. A standard curve was generated by fitting a four-parameter logistic function on a log-log scale, with concentration plotted on the x-axis and OD values on the y-axis.

All data were analyzed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). The statistical analysis was performed by a one-way analysis of variance (ANOVA). Values were shown as mean $\pm$ standard deviation ($\bar{x} \pm s$). Differences were considered significant if $P < 0.05$.

Results

Identification of Intersecting Targets Between Biapenem and Severe Pneumonia

Using the GeneCards database with "severe pneumonia" as the keyword, a total of 1,952 disease-associated targets were retrieved, along with 275 biapenem-related drug targets. The biapenem-related targets and severe pneumonia-related targets were imported into the Venny 2.1 online tool to generate a drug–disease target Venn diagram (Fig. 1a), yielding 78 intersecting targets. The intersecting targets and the corresponding disease information were imported into Cytoscape 3.8.0 software to construct a "drug–target–disease" network (Fig. 1b).

The 78 intersecting targets were submitted to the STRING database to construct a PPI network (Fig. 1c), and the resulting network data were imported into Cytoscape 3.8.0 software to identify core targets based on degree values. In the PPI network, the top 10 targets ranked by degree were AKT1, EGFR, PPARG, JAK2, KIT, PTPN11, ACE, PLG, and SYK, which were predicted to be the key target proteins mediating the beneficial effects of biapenem in severe pneumonia.

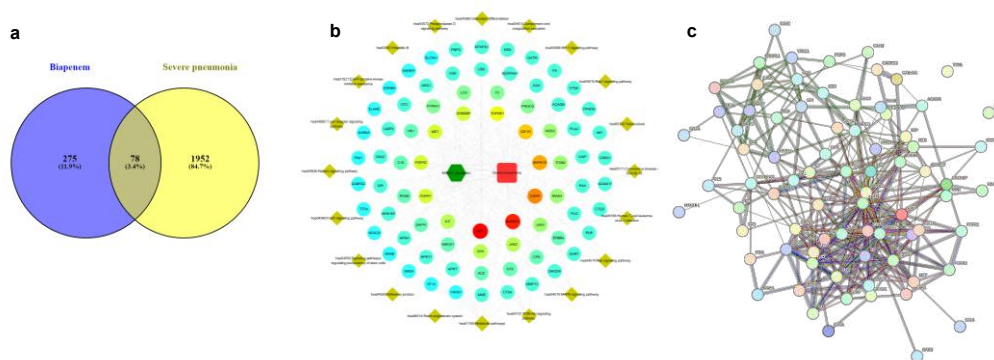


Figure 1 Network pharmacology analysis exploring the major targets of biapenem in the treatment of severe pneumonia (a) biapenem–severe pneumonia Venn diagram.

Biapenem has 275 targets, severe pneumonia has 1,952 targets, and the intersection between them contains 78 targets (b) Biapenem–severe pneumonia network diagram. The disease pathways include the PI3K-Akt signaling pathway and the EGFR tyrosine kinase inhibitor resistance signaling pathway, among others (c) PPI network of protein–protein interactions

Biapenem Treats Severe Pneumonia via Protein Phosphorylation and Related Signaling Pathways

The 78 intersecting targets were imported into the DAVID database for GO enrichment analysis (Fig. 2a). The GO enrichment analysis was categorized into three major domains: biological process (BP), cellular component (CC), and molecular function (MF). The results revealed that biapenem exerts its therapeutic effects on severe pneumonia primarily through biological processes including positive regulation of MAPK cascade, protein autophosphorylation, proteolysis, positive regulation of PI3K/PKB signal transduction, peptidyl-tyrosine phosphorylation, negative regulation of apoptotic process, positive regulation of signaling, positive regulation of cell communication, protein phosphorylation, and the epidermal growth factor receptor signaling pathway. The associated cellular components included cytosol, receptor complex, membrane raft, extracellular space, cell surface, extracellular region, extracellular exosome, cytoplasm, plasma membrane, and ficolin-1-rich granule lumen. The enriched molecular functions comprised kinase activity, protein kinase activity, protein tyrosine kinase activity, transferase activity, ATP binding, nucleotide binding, peptidase activity, endopeptidase

activity, transmembrane receptor protein tyrosine kinase activity, and serine-type endopeptidase activity.

KEGG enrichment analysis was performed to identify the target signaling pathways. With a threshold of P value < 0.01, pathways were ranked by gene count and visualized using a bubble plot (Fig. 2b). The analysis revealed that multiple pathways were associated with cancer and intercellular signal transduction, including the HIF-1 signaling pathway, PI3K-Akt signaling pathway, complement and coagulation cascades, Ras signaling pathway, MAPK signaling pathway, EGFR tyrosine kinase inhibitor resistance, human T-cell leukemia virus 1 infection, tuberculosis, coronavirus disease—COVID-19, osteoclast differentiation, phospholipase D signaling pathway, Rap1 signaling pathway, hepatitis B, T cell receptor signaling pathway, relaxin signaling pathway, FoxO signaling pathway, renin-angiotensin system, adherens junction, metabolic pathways, and signaling pathways regulating pluripotency of stem cells.

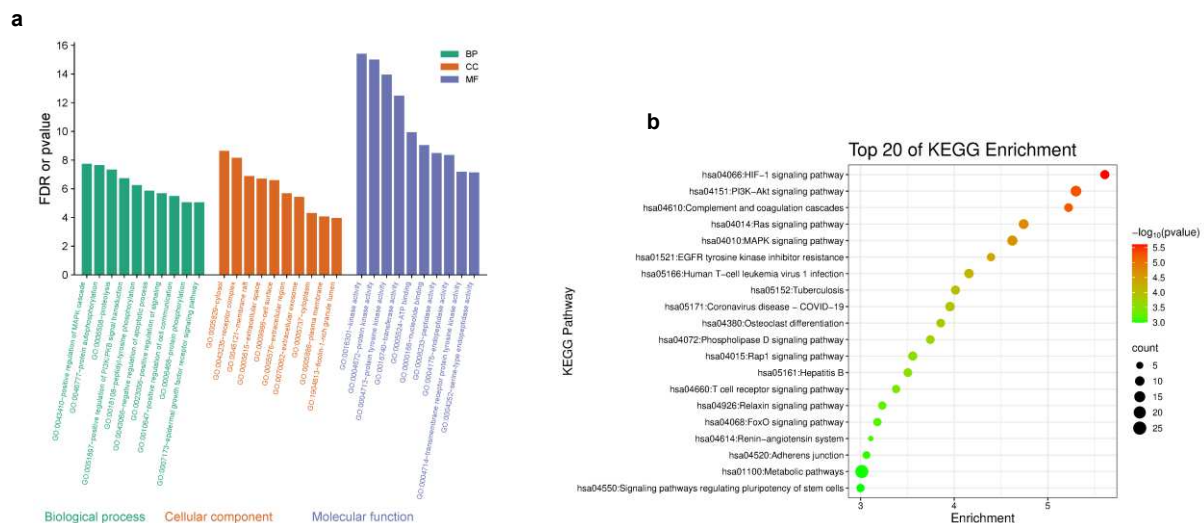


Figure 2 GO and KEGG pathway enrichment analyses of biapenem (a) GO analysis.

The therapeutic effects of biapenem on severe pneumonia may be achieved through

positive regulation of the MAPK cascade and regulation of protein phosphorylation (b) KEGG pathway analysis. The mechanisms underlying the effects of biapenem may be associated with signaling pathways such as the HIF-1 signaling pathway and the PI3K-Akt signaling pathway. KEGG images are used with permission from Kanehisa Laboratories.

AKT1 Is the Primary Target of Biapenem in the Treatment of Severe Pneumonia

Based on the results of the "Biapenem–target–severe pneumonia" network analysis, molecular docking analysis of biapenem was performed in this study. Docking simulations were conducted using the "CDOCKER" module in Discovery Studio 2019 software, and the CDOCKER interaction energy (–CIE value) was used to evaluate the binding affinity between the active compound and target proteins, with higher –CIE values indicating stronger binding activity. Specifically, the –CIE values between biapenem and the selected key targets (AKT1, TGFBR1, MAP2K1, FGFR2, EGFR, FGFR1, IGF1R, MAPK10, KIT, and CREBBP) were calculated to assess the structural stability of the resulting complexes. Higher –CIE values predict greater stability of the biapenem–target protein complex, potentially enhancing its therapeutic potential. The docking results showed that biapenem exhibited the highest –CIE values with AKT1 and TGFBR1 (both –8.6), followed by MAP2K1 (–7.6), FGFR2 (–7.4), EGFR (–7.2), FGFR1 (–7.2), IGF1R (–6.9), MAPK10 (–6.9), KIT (–6.7), and CREBBP (–5.6).

Further analysis revealed that biapenem primarily binds to threonine and serine residues of the core targets through hydrogen bonds, π interactions, and van der Waals forces, thereby enhancing the stability of the complexes (Fig. 3). Since the KEGG results

indicated that the targets of biapenem were associated with the HIF-1 signaling pathway and the PI3K-AKT signaling pathway, and given that biapenem could form a stable complex with AKT1, the subsequent investigation in this study focused on the TLR4/NF- κ B/PI3K-AKT signaling pathway to explore the mechanism of action of biapenem in severe pneumonia.

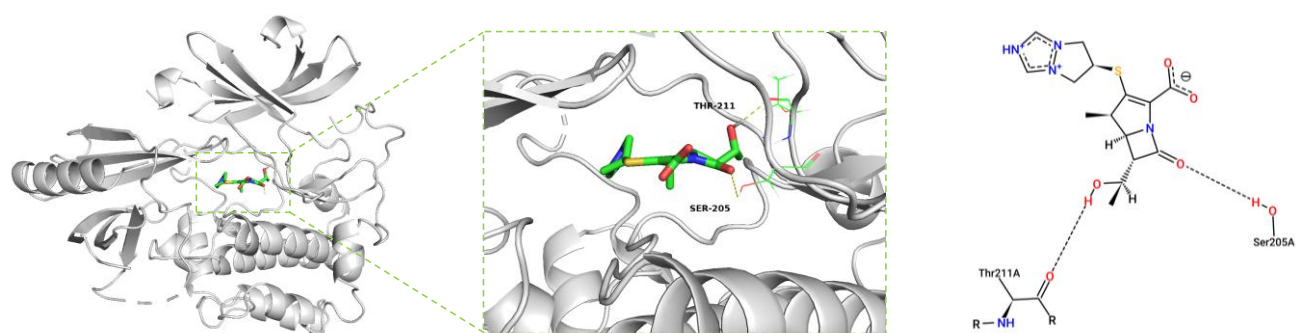


Figure 3 3D conformation of the complex formed between AKT1 and biapenem

Determination of Biapenem Intervention Concentrations

First, the LPS induction concentration was determined. A549 cells were treated with LPS at 1, 5, 10, and 20 μ g/mL, and cell viability was assessed using the CCK-8 assay. As shown in Fig. 4a, differences in cell viability began to emerge at an LPS concentration of 5 μ g/mL; at 10 μ g/mL, the mean cell survival rate was 77.42%, and at 20 μ g/mL, it was 60.67%, both showing significant differences. Since this study required an LPS concentration that reduced cell viability to approximately 70% to serve as the inflammatory model induction concentration, 10 μ g/mL LPS was selected for all subsequent experiments. Second, the biapenem intervention concentration was determined. Biapenem at concentrations of 10, 25, 50, 100, and 200 μ g/mL was tested. As shown in Fig. 4b, treatment with 25 μ g/mL biapenem had no significant effect on A549

cell viability; therefore, the maximum intervention concentration of biapenem was set at 25 $\mu\text{g/mL}$. Using a two-fold serial dilution method, the medium concentration of biapenem was set at 12.5 $\mu\text{g/mL}$ and the low concentration at 6.25 $\mu\text{g/mL}$ for subsequent experimental grouping. Finally, the dexamethasone intervention concentration was determined. As shown in Fig. 4c, A549 cells stimulated with LPS were treated with dexamethasone at 0.1, 1, and 10 $\mu\text{mol/L}$. Dexamethasone at 0.1 $\mu\text{mol/L}$ failed to restore cell viability, while 10 $\mu\text{mol/L}$ dexamethasone exhibited certain cytotoxicity, resulting in decreased cell viability. In contrast, 1 $\mu\text{mol/L}$ dexamethasone restored cell viability following LPS stimulation without exerting adverse effects on cells. Therefore, 1 $\mu\text{mol/L}$ dexamethasone was used as the positive control in subsequent experiments.

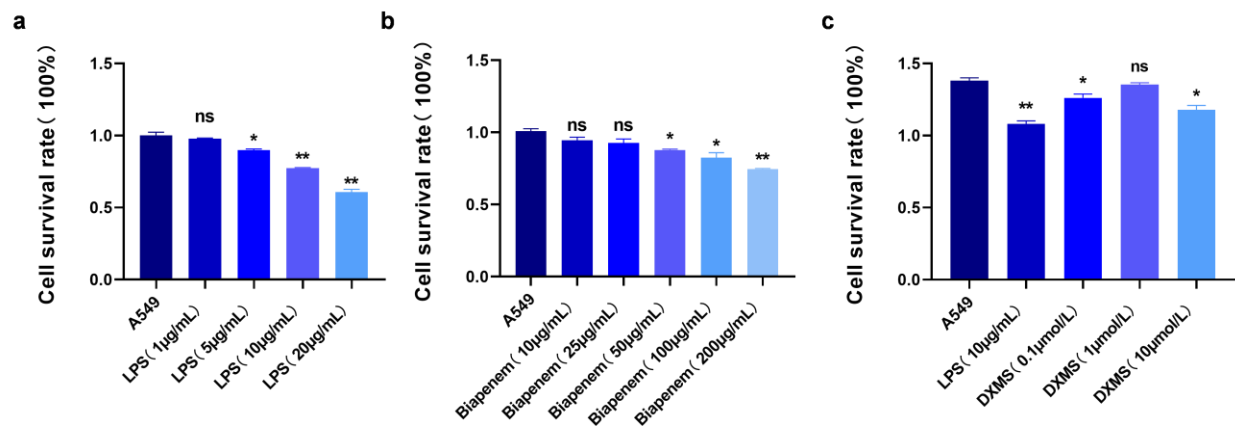


Figure 4 Determination of intervention concentrations in A549 cells (a) Determination of the LPS intervention concentration. Treatment with 10 $\mu\text{g/mL}$ LPS induced an inflammatory response in A549 cells (b) Determination of the biapenem intervention concentration. A concentration of 25 $\mu\text{g/mL}$ was identified as the optimal intervention concentration of biapenem (c) Determination of the dexamethasone intervention concentration. A concentration of 1 $\mu\text{mol/L}$ was identified as the optimal intervention

concentration of dexamethasone. Statistical analysis was performed using one-way ANOVA, with the A549 group serving as the control. $*p < 0.05$, $**p < 0.01$

Biapenem Reduces the Expression of Inflammatory Cytokines and Pathway

Proteins

To investigate the inhibitory effects of biapenem on LPS-induced inflammatory responses in A549 cells and its regulation of the TLR4/NF- κ B/PI3K-AKT signaling pathway, Western blotting was performed to detect the expression levels of relevant proteins in each group (Fig. 5), full-length blots are presented in supplementary date 1. Compared with the control group, the protein expression levels of p-AKT, the p-AKT/AKT ratio, p65, p-P65, and TLR4 were significantly elevated in the LPS-induced model group, whereas AKT expression remained essentially unaffected. Compared with the model group, high-concentration biapenem intervention significantly reduced the protein expression levels of p-AKT, the p-AKT/AKT ratio, p-P65, and TLR4, consistent with the trend observed in the dexamethasone positive control group. In addition, medium-concentration biapenem intervention also significantly decreased the expression levels of p-AKT, the p-AKT/AKT ratio, and p-P65, while low-concentration biapenem intervention significantly reduced only p-p65 expression; p-AKT, p65, and TLR4 exhibited only downward trends without statistical significance. These results demonstrated that biapenem exhibited dose-dependent hierarchical inhibition of the LPS-induced inflammatory signaling pathway. The minimum effective concentration of its anti-inflammatory action significantly targeted p-P65, indicating that this site represents the most drug-sensitive signaling node. At medium concentrations, biapenem further blocked

p-AKT, achieving simultaneous interference with both the PI3K/AKT and NF- κ B pathways and enhancing anti-inflammatory efficacy by disrupting the synergistic amplification between the two pathways. Only at high concentrations was TLR4 receptor protein expression significantly downregulated, suggesting the existence of a relatively high pharmacological threshold for inhibition of the upstream receptor. The inhibitory profile of high-concentration biapenem was highly consistent with that of dexamethasone, indicating its potential for broad-spectrum inflammatory regulation.

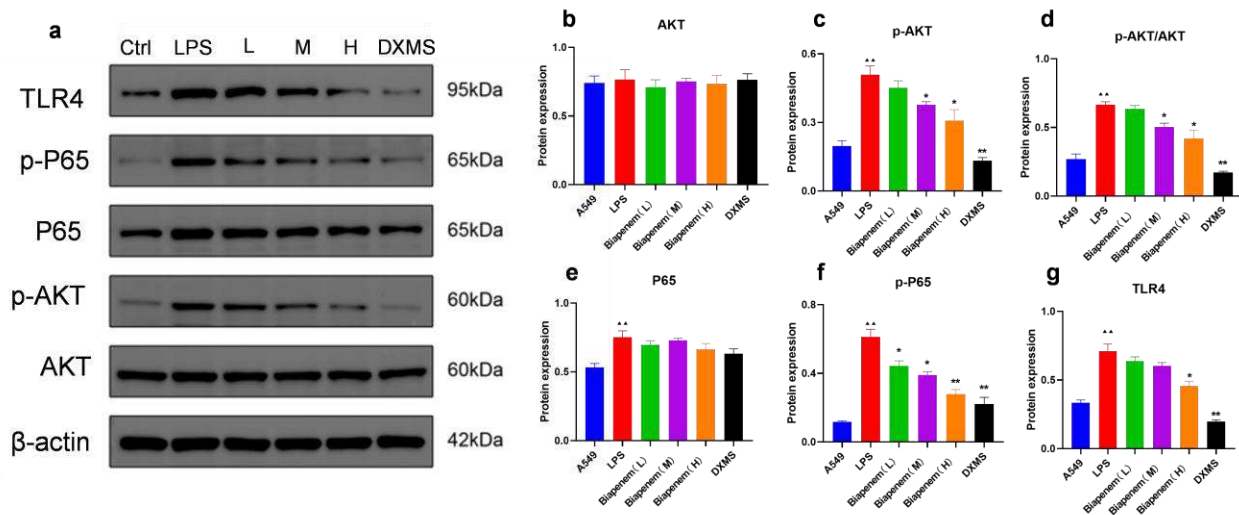


Figure 5 Effects of biapenem on the expression of inflammatory cytokines and TLR4/NF- κ B/PI3K-AKT pathway proteins in LPS-stimulated A549 cells (a) Representative Western blot images. Cropped from different gels (b) Expression levels of AKT protein in each group (c) Expression levels of p-AKT protein in each group (d) p-AKT/AKT ratio in each group (e) Expression levels of P65 protein in each group (f) Expression levels of p-P65 protein in each group (g) Expression levels of TLR4 protein in each group. Statistical analysis was performed using one-way ANOVA, with the A549

group serving as the control: $\Delta p < 0.05$, $\Delta\Delta p < 0.01$; and the LPS group serving as the control: $*p < 0.05$, $**p < 0.01$

Biapenem Reduces the mRNA Expression of Inflammatory Cytokines

To further investigate the effects of biapenem on the TLR4/NF- κ B/PI3K-AKT signaling pathway at the mRNA level, qPCR was performed to detect the expression levels of relevant mRNAs in each group. The primer sequences are listed in Table 1, and the results are shown in Fig. 6. Compared with the control group, the mRNA expression levels of TLR4 and P65 were significantly elevated in the LPS-induced model group, along with significantly increased mRNA expression of the inflammatory cytokines IL-1 β , IL-6, and TNF- α , confirming that LPS markedly induced inflammatory responses. Compared with the model group, intervention with low, medium, and high concentrations of biapenem significantly reduced the mRNA expression levels of TLR4, NF- κ B p65, IL-1 β , IL-6, and TNF- α , with the inhibitory effects on mRNA expression exhibiting a stepwise enhancement, consistent with the trend observed in the dexamethasone positive control group. The qPCR results further confirmed that biapenem exerted broad-spectrum and stepwise inhibitory effects on LPS-induced inflammatory responses at the transcriptional level. All three concentrations of biapenem significantly downregulated the mRNA expression of TLR4, NF- κ B p65, IL-1 β , IL-6, and TNF- α , with the inhibitory effects increasing in a gradient manner as the concentration increased, demonstrating a strict dose-dependent relationship. Notably, in contrast to the protein level, where TLR4 was significantly reduced only at high concentrations, biapenem effectively inhibited TLR4 transcription at the mRNA level even at low concentrations, suggesting that the threshold

for transcriptional regulation by biapenem is lower and that it may block the reproduction of inflammatory signaling at its source by directly interfering with the transcriptional initiation activity of NF- κ B. This transcriptional inhibitory profile was highly consistent with that of dexamethasone, further establishing biapenem as a molecular candidate with dual anti-infective and transcriptional anti-inflammatory functions, thereby providing transcriptomic evidence for its precise application in hyperinflammatory states.

Table 1 Primer information

mRNA	Base composition	length	NCBI Reference Sequence
TLR4	AAAATCCCCGACAACCTCCC TGTCTGGATTTACACCTGGA	138	NM_138554.5
RELA (NF- κ B p65)	GCTGCATCCACAGTTTCCAG TCCCCACGCTGCTCTTCTAT	130	NM_021975.4
AKT1	ATGGACAGGGAGAGCAAACG CTGGCCACAGCCTCTGATG	134	NM_001382430.1
IL-1 β	CCAAACCTCTTCGAGGCACA GCTGCTTCAGACACTTGAGC	85	NM_000576.3
IL-6	CCGGGAACGAAAGAGAAGCTC ACCGAAGGCGCTTGTGGAG	75	NM_000600.5
TNF- α	GCCCATGTTGTAGCAAACCC TGAGGTACAGGCCCTCTGAT	133	NM_000594.4
β -actin	CGACAGGATGCAGAAGGAGA CGATCCACACGGAGTACTTG	95	NM_001101.5

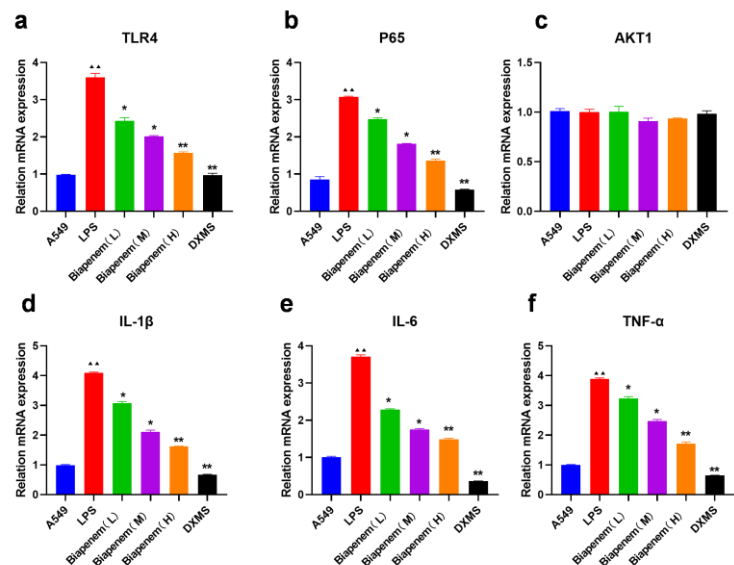


Figure 6 Effects of biapenem on the mRNA expression of inflammatory cytokines and TLR4/NF-κB/PI3K-AKT pathway components in LPS-stimulated A549 cells (a) mRNA expression levels of TLR4 in each group (b) mRNA expression levels of NF-κB p65 in each group (c) mRNA expression levels of AKT1 in each group (d) mRNA expression levels of IL-1β in each group (e) mRNA expression levels of IL-6 in each group (f) mRNA expression levels of TNF-α in each group. Statistical analysis was performed using one-way ANOVA, with the A549 group serving as the control: ▲ $p < 0.05$, ▲▲ $p < 0.01$; and the LPS group serving as the control: * $p < 0.05$, ** $p < 0.01$

Biapenem Attenuates the Inflammation at the Transcriptional Initiation Stage

In this study, ELISA was further employed to detect changes in IL-1β, IL-6, and TNF-α secretion following biapenem intervention, and the results are shown in Fig. 7. Compared with the LPS model group, all three concentrations of biapenem (low, medium, and high) significantly reduced the protein secretion levels of IL-1β, IL-6, and

TNF- α in the cell culture supernatant, with the inhibitory effects progressively enhanced as the concentration increased, demonstrating a clear concentration-dependent stepwise effect.

Notably, the trends in secreted protein levels observed by ELISA were highly consistent with the mRNA expression levels detected by qPCR. Both assays demonstrated significant inhibition at low concentrations, with further enhancement of the inhibitory effects at medium and high concentrations, and the relative inhibition magnitudes of the three inflammatory cytokines were completely consistent. This concordance rules out the possibility that biapenem merely affects mRNA stability or only interferes with protein secretion, demonstrating that biapenem initiates its anti-inflammatory program at the stage of gene transcriptional initiation.

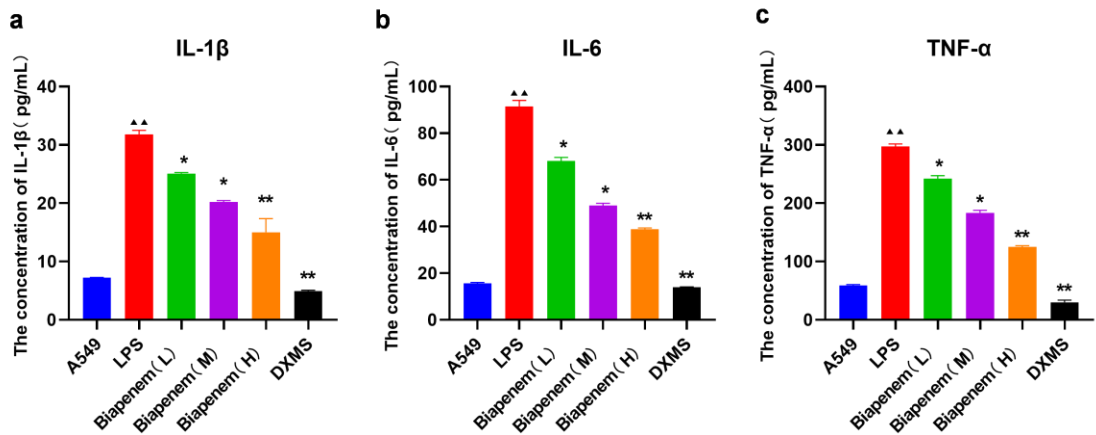


Figure 7 Effects of biapenem on inflammatory cytokines in LPS-stimulated A549 cells (a) Concentrations of IL-1 β in each group (b) Concentrations of IL-6 in each group (c) Concentrations of TNF- α in each group. Statistical analysis was performed using one-way ANOVA, with the A549 group serving as the control: $\Delta p < 0.05$, $\Delta\Delta p < 0.01$; and the LPS group serving as the control: $*p < 0.05$, $**p < 0.01$

Discussion

In this study, we systematically investigated the regulatory effects of the carbapenem antibiotic biapenem on LPS-induced inflammatory responses and the underlying molecular mechanisms through in vitro cell experiments. Integrating the results from Western blotting, qPCR, and ELISA, we found that biapenem inhibited LPS-triggered inflammatory responses in a concentration-dependent manner across three hierarchical levels: transcription, translation, and secretion. Its targets encompassed the complete signaling axis, spanning from the membrane receptor TLR4 to the intracellular signaling molecules p-AKT and p-P65, and further to the downstream effector cytokines IL-1 β , IL-6, and TNF- α .

LPS, as the major component of the outer membrane of Gram-negative bacteria, is a key molecular pattern recognized by the innate immune system (Singh et al., 2022). Upon binding to the TLR4 receptor on the cell membrane, LPS recruits adaptor proteins such as MyD88, leading to activation of the downstream IKK complex, which in turn phosphorylates I κ B α and targets it for degradation, releasing NF- κ B dimers (primarily p65/p50) for nuclear translocation (Li et al., 2024) and initiating target gene transcription (Wang et al., 2017). In the present study, both the mRNA and protein levels of TLR4 and p65 were significantly elevated following LPS stimulation, accompanied by enhanced p-p65 phosphorylation, confirming successful activation of this pathway.

Notably, biapenem significantly inhibited p-p65 phosphorylation and the transcription and secretion of inflammatory cytokines even at low concentrations, whereas significant inhibition of TLR4 and p-AKT required medium and high concentrations. This differential

sensitivity suggests that p65 phosphorylation represents the most sensitive molecular target of biapenem. From the perspective of signaling architecture, p65, as the core subunit of the NF- κ B transcriptional complex, undergoes phosphorylation that serves as the final common pathway for inflammatory cytokine transcription (Cui et al., 2022, McKee et al., 2021). Therefore, precise targeting of p-p65 by low concentrations of the drug is sufficient to initiate the downstream anti-inflammatory program. This positioning is consistent with previous reports demonstrating that biapenem alleviates PM2.5-induced lung injury by inhibiting the TLR4/NF- κ B pathway (Lee et al., 2020).

We observed that LPS stimulation significantly increased p-AKT levels and the p-AKT/AKT ratio, while total AKT levels remained unchanged, suggesting that LPS activates the PI3K/AKT pathway primarily through phosphorylation modification. Medium and high concentrations of biapenem significantly reduced p-AKT levels and the p-AKT/AKT ratio, indicating that the drug's effects extend from sole inhibition of the NF- κ B pathway to simultaneous blockade of the PI3K/AKT axis. As a core hub integrating cell survival, metabolism, and inflammatory signaling, inhibition of AKT phosphorylation not only attenuates the transactivation capacity of NF- κ B but may also synergistically enhance anti-inflammatory effects by reducing the activation of downstream effectors such as mTOR (Matsubara et al., 2020). This pattern of anti-inflammatory axis modulation explains why medium and high concentrations of the drug more thoroughly suppress the inflammatory cytokine storm.

The high degree of consistency between ELISA and qPCR results represents another key finding of this study. At low, medium, and high concentrations, the mRNA

expression and protein secretion of IL-1 β , IL-6, and TNF- α exhibited completely parallel downward trends. This finding rules out the possibility that the drug merely affects mRNA stability or only interferes with protein secretion, demonstrating that biapenem initiates its anti-inflammatory program at the stage of gene transcriptional initiation. Moreover, the three distinct inflammatory cytokines exhibited consistent concentration-dependent responses across both detection methods, indicating that biapenem exerts its anti-inflammatory effects through systematic regulation of upstream common signaling pathways rather than selective intervention targeting a specific cytokine. Previous studies have shown that biapenem significantly inhibits HMGB1 release, reduces vascular hyperpermeability and tissue injury, and decreases sepsis mortality by 50% in LPS-activated human umbilical vein endothelial cells and in a cecal ligation and puncture (CLP)-induced sepsis mouse model (Kim et al., 2021). These in vivo and in vitro evidence mutually corroborate the findings at the cytokine level in the present study, collectively establishing a molecular evidence chain supporting the dual anti-infective and anti-inflammatory properties of biapenem.

The inhibitory effects of high-concentration biapenem on various targets of the TLR4/NF- κ B/PI3K-AKT pathway were highly consistent with those of the positive control dexamethasone. As a classical potent glucocorticoid, dexamethasone exerts its anti-inflammatory effects primarily through glucocorticoid receptor-mediated transcriptional repression, interfering with multiple components of the LPS-TLR4/NF- κ B pathway (Świerczek and Jusko, 2023). Previous studies have also confirmed that dexamethasone inhibits NF- κ B activation and inflammatory cytokine secretion in the TLR4 signaling

pathway (Bonin et al., 2013). However, although glucocorticoids such as dexamethasone possess excellent anti-inflammatory efficacy, their severe adverse effects—including hyperglycemia, immunosuppression leading to secondary infections, myopathy, and neuropsychiatric symptoms—greatly limit their widespread application in patients with sepsis (Giles et al., 2018). As a β -lactam antibiotic, biapenem, despite having a mechanism of action distinct from that of corticosteroids, can achieve a comparable breadth and depth of modulation at the signaling network level. In fact, the immunomodulatory effect of biapenem is not an isolated phenomenon among carbapenems. In recent years, increasing evidence has demonstrated that β -lactam antibiotics possess direct anti-inflammatory activities. Ceftriaxone significantly downregulates TNF- α and upregulates IL-10 in a rat carrageenan-induced inflammation model (Ochoa-Aguilar et al., 2018), meropenem reduces circulating IL-6 levels by inhibiting NF- κ B activation in a CLP sepsis model (Giamarellos-Bourboulis et al., 2006), and imipenem exhibits neuroprotective effects independent of its antibacterial activity in a meningitis model (Herve et al., 2004). Nevertheless, significant differences exist among members of the carbapenem class. Compared with meropenem or imipenem, biapenem exhibits higher stability against renal dehydropeptidase-I, obviating the need for co-administration with cilastatin, and demonstrates superior penetration into lung tissue and alveolar lining fluid (Yamada et al., 2008) , endowing it with both pharmacokinetic advantages and anti-inflammatory regulatory potential in the settings of severe pneumonia and ventilator-associated pneumonia. The mechanistic depth of this study lies in precisely localizing the site of action of biapenem to the TLR4/NF- κ B/PI3K-AKT axis

and, for the first time, revealing a hierarchical pattern using a unified dataset: low concentrations target p-p65, medium concentrations achieve dual-axis co-inhibition, and high concentrations attenuate TLR4 expression. This represents a relatively complete evidence chain among studies on the anti-inflammatory mechanisms of carbapenems. The findings suggest that β -lactam antibiotics may possess shared anti-inflammatory regulatory potential, offering a novel perspective for their application in scenarios of infection complicated by excessive inflammation, and providing direct molecular pharmacological evidence for precise dose stratification of biapenem in the treatment of severe inflammatory conditions. Particularly in critical scenarios such as sepsis, where simultaneous control of pathogens and excessive inflammation is required, biapenem may offer a safer alternative to corticosteroids—one that integrates anti-infective efficacy with concentration-adjustable inflammatory regulatory capacity (Chen et al., 2024). Moreover, as a carbapenem antibiotic, its long-term use risks (such as immunosuppression and metabolic disturbances) are more manageable than those associated with corticosteroids (Shuto et al., 2024).

This study has several limitations. First, the current evidence is derived exclusively from an in vitro cell model; whether the threshold effects on TLR4 and the hierarchical inhibitory pattern remain valid in the in vivo circulation or tissue microenvironment requires verification through animal experiments. Second, whether biapenem directly binds to the TLR4/MD-2 complex or indirectly interferes with signal transduction by affecting cell wall components remains to be confirmed through molecular docking and other techniques. Finally, whether the inhibitory effect of biapenem on the PI3K/AKT

pathway is mediated by direct action on PI3K kinase or occurs indirectly through attenuation of upstream TLR4 signaling requires further elucidation. Future research may focus on the molecular binding mode between biapenem and TLR4, as well as the pharmacodynamic–pharmacokinetic relationship in animal models of sepsis, to facilitate its clinical translation.

Conclusions

This study demonstrates that Biapenem exerts a anti-inflammatory effect in LPS-stimulated macrophages by suppressing the TLR4/NF-κB/PI3K-AKT signaling axis. It directly targets p-P65 to initiate transcriptional repression of pro-inflammatory cytokines, achieves dual-axis blockade of both NF-κB and PI3K/AKT pathways and diminishes TLR4 expression at the source. These findings reposition Biapenem as a dual-function molecule with both antimicrobial and anti-inflammatory properties, offering a potential therapeutic strategy for severe inflammatory conditions.

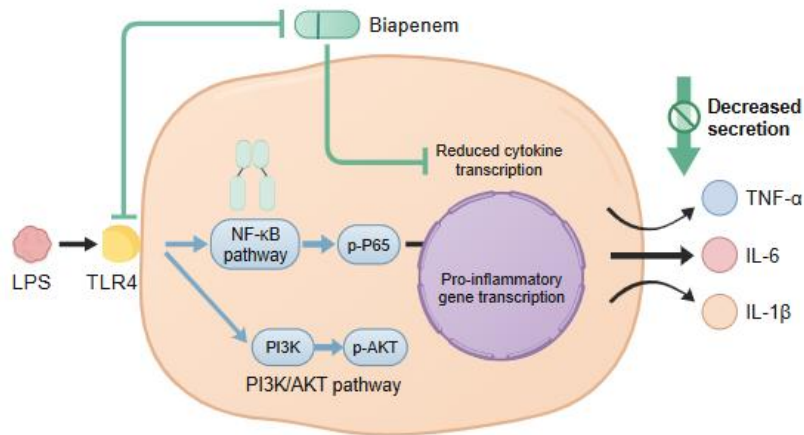


Figure 8 Biapenem exerts concentration-dependent anti-inflammatory effects by modulating the TLR4/NF-κB/PI3K-AKT signaling axis.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Enjing Zhang and Xinwei Li. The first draft of the manuscript was written by Xinwei Li and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets used during the current study are available from the corresponding author on reasonable request.

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