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*Original Article*

# TRAIL-mediated armA upregulation enhances the drug resistance of *Klebsiella pneumoniae* by activating the PI3K/AKT/mTOR signaling pathway

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**Abstract:** Objective: This study aimed to investigate the roles and mechanisms of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the drug resistance of *Klebsiella pneumoniae*, focusing on its regulation of the aminoglycoside resistance methylase (armA) and the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. Methods: A549 cells were infected with drug-resistant *Klebsiella pneumoniae* and treated with meropenem. TRAIL overexpression and knockdown were performed using plasmids and small interfering RNA, respectively. Cell viability, apoptosis, and the levels of inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were assessed. The mRNA expression of armA was examined using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression of key proteins in the PI3K/AKT/mTOR pathway was evaluated using western blots. Results: Drug-resistant *Klebsiella pneumoniae* infection reduced A549 cell viability, promoted apoptosis, and increased TNF-α, IL-6, and IL-1β levels. Meropenem treatment failed to reverse these effects, confirming the drug resistance. TRAIL overexpression exacerbated *Klebsiella pneumoniae* infection-induced viability inhibition, apoptosis, and inflammation, suggesting that TRAIL enhances the drug resistance of *Klebsiella pneumoniae*. In contrast, TRAIL knockdown showed the opposite results. TRAIL overexpression upregulated armA expression and activated the PI3K/AKT/mTOR pathway, but armA inhibition reversed TRAIL-mediated drug resistance and PI3K/AKT/mTOR activation. Conclusion: TRAIL-mediated armA upregulation enhanced the drug resistance of *Klebsiella pneumoniae* by activating the PI3K/AKT/mTOR signaling pathway. These findings provide new insight into the drug resistance mechanisms of *Klebsiella pneumoniae*.

**Keywords:** *Klebsiella pneumoniae*, drug resistance, TRAIL, armA, PI3K/AKT/mTOR signaling pathway

## Introduction

In recent years, the escalating challenge of antibiotic resistance has emerged in global medicine and biomedicine. The treatment landscape for bacterial infections has gotten complex, as an expanding repertoire of pathogens develop resistance to currently available antibiotics, rendering many conventional therapies ineffective [1-3]. Among these multidrug-resistant pathogens, *Klebsiella pneumoniae*, a Gram-negative bacteria responsible for pneumonia, bloodstream infections, and other severe infections, has garnered attention due to its rapidly evolving drug resistance profile [4, 5]. The alarming rise in *Klebsiella* resistance patterns poses a substantial threat to global public health [6]. Therefore, it is urgent to elucidate its resistance mechanisms to develop novel therapeutic strategies and combat resistant infections.

The aminoglycoside resistance methylase (armA) gene, encoding a crucial ribosome-modifying methyltransferase, plays a pivotal role in bacterial physiology and antibiotic resistance [7-9]. This enzyme catalyzes the transfer of methyl groups to specific adenine residues in

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16S rRNA, thereby modulating bacterial protein translation and gene expression [10, 11]. While prior evidence has emphasized the crucial roles of armA methylase gene in antibiotic resistance, the precise molecular mechanisms underlying its contribution to *Klebsiella* resistance remain poorly elucidated.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a TNF superfamily member, is known for inducing apoptosis in cancer cells [12, 13]. Evidence indicates its critical involvement in modulating host immune responses to bacterial infections, including those caused by *Klebsiella pneumoniae* [14, 15]. Notably, TRAIL has emerged as a target in a rapid host-protein test for differentiating bacterial from viral infections [16]. Moreover, TRAIL-targeted therapy promoted macrophage apoptosis and alleviated pneumococcal pneumonia in mice [17]. TRAIL-encapsulated nanogel exhibited significant anti-inflammatory effects in *Klebsiella pneumoniae*-induced sepsis [15]. However, the precise roles of TRAIL in regulating the armA methylase gene and its contribution to *Klebsiella* drug resistance remain uncertain.

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is a central coordinator of cellular survival, proliferation, and metabolic regulation [18-20]. Its pathologic activation has been extensively documented in conditions including cancer, inflammatory disease, and infectious disease [19-21]. However, the specific involvement of the PI3K/AKT/mTOR signaling pathway in *Klebsiella* drug resistance, and whether it interacts with the armA methylase gene, remains largely unexplored.

The purpose of this study is to elucidate the roles and mechanisms of TRAIL in drug resistance of *Klebsiella pneumoniae*, with special attention to its regulation in armA expression and the PI3K/AKT/mTOR signaling pathway. The findings should deepen our comprehension of *Klebsiella* drug resistance, and provide new ideas for future antibacterial drug development.

## Materials and methods

### *Culture of drug-resistant Klebsiella strain*

The drug-resistant *Klebsiella* strain obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) was initially retrieved from a cryopreserved bacterial stock. The bacterial suspension was streaked onto Luria-Bertani (LB) agar plates containing antibiotics and incubated overnight at 37°C for 18-24 h to facilitate the formation of individual bacterial colonies. A single colony was then aseptically selected and cultured in liquid LB medium (Gibco, USA) containing corresponding antibiotics with shaking (200 rpm) overnight at 37°C until reaching the logarithmic growth phase. These log-phase bacterial cultures were used for subsequent experiments.

### *Cell line culture*

To simulate the host cell environment during *Klebsiella* infection, a human lung epithelial cell line (A549 cells) was obtained from ATCC (Manassas, VA, USA). A549 cells were rapid-thawed at 37°C and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic solution under 5% $CO_2$ at 37°C. The culture medium was replaced every 2 days. After reaching 80% confluence, cells were harvested and subjected to further studies, including infection assays or cell proliferation studies.

### *Bacterial infection model and antibiotic treatment*

A549 cells were plated in 96-well plates and incubated at 37°C in 5% $CO_2$. Then, 1 mL of log-phase bacterial culture was centrifuged and resuspended in phosphate buffered saline (PBS) to adjust to $1\times10^8$ CFU/mL. Cells were collected and incubated with 100 μL of bacterial suspension in culture medium at 37°C in 5% $CO_2$ for 2 h. The bacteria-containing medium was then changed to a medium containing 100 μg/mL gentamicin, followed by culture at 37°C for 24 h to eliminate extracellular bacteria. For antibiotic treatment, A549 cells were treated with 16 μg/mL meropenem (MEM; Sigma-Aldrich, St. Louis, MO, USA), a carbapenem antibiotic, for 24 h.

### *Cell transfection*

TRAIL overexpression plasmids (OE-TRAIL) and small interfering RNAs targeting TRAIL (sh-TRAIL) or armA (si-armA) were sourced from RiboBio (Guangzhou, China). They were transfect-

ed into A549 cells using Lipofectamine™ 3000 (Invitrogen, USA). The siRNA sequences were: si-TRAIL (5'-TCT GTG TGG CTG TAA CTT A-3'), si-armA (5'-GGAAGTTAAAGACGACGATA-3').

*Cell counting Kit-8 (CCK-8) assay*

A549 cells were seeded into 96-well plates at a concentration of $5\times10^3$ cells. After 24-hour incubation, the culture medium was removed and replaced with 100 μL of fresh DMEM containing 10% CCK-8 reagent (Dojindo, Japan) to incubate at 37°C for 2 h. The absorbance at 450 nm was measured using a microplate reader (BioTek, USA).

*Cell apoptosis*

A549 cells were stained with the Annexin V-FITC and propidium iodide (BD Bioscience, USA) for 15 min in dark. Subsequently, the apoptotic cells were determined using flow cytometry (BD Bioscience, USA).

*Determination of inflammatory cytokines*

The levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, Wuhan, China).

*Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

Initially, total RNAs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then were reverse-transcribed into cDNA through a reverse transcription reaction. RT-qPCR was subsequently conducted using SYBR Green Master Mix (Applied Biosystems, USA), and normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: armA (forward, 5'-AAA GTA CAA TCA GGG GCA GTT-3' and reverse, 5'-TCG TCG TCT TTA ACT TCC CAA-3'), GAPDH (forward, 5'-TCA CTG TTC TCT CCC TCC GC-3' and reverse, 5'-TAC GAC CAA ATC CGT TGA CTC C-3').

*Western blot analysis*

Total proteins were extracted and separated by SDS-PAGE. Subsequently, the membranes were incubated with specific primary antibodies at 4°C overnight and secondary antibody (ab6702, Abcam) for 2 h at room temperature. The protein signals were developed with enhanced chemiluminescence reagent and analyzed with ImageJ software. The primary antibodies used were: TRAIL (ab42121, Abcam), phosphorylated PI3K (p-PI3K; ab138364, Abcam), phosphorylated AKT (p-AKT; ab38449, Abcam), phosphorylated mTOR (p-mTOR; ab109268, Abcam), and β-actin (ab8226, Abcam).

*Data analysis*

Data were expressed as mean ± standard deviation (SD), and analyzed statistically using SPSS 26.0. The normal distribution of data was verified by the Shapiro-Wilk test, and the homogeneity of variances was verified by the Levene's test. Non-parametric tests (Kruskal-Wallis test/Mann-Whitney test) were used if data were not normally distributed or variances were not homogeneous. Two-group analysis were performed using Student's *t*-test, and multi-group analysis was compared by one-way analysis of variance (ANOVA) followed by Tukey-Kramer correction. Differences were considered significant at the $P < 0.05$ level.

## Results

*Drug-resistant Klebsiella pneumoniae infection promoted apoptosis and inflammation in A549 cells*

A drug-resistant *Klebsiella pneumoniae*-infected A549 cell model was prepared and treated with meropenem, followed by determination of cell viability, apoptosis, and inflammatory cytokine levels. Our results demonstrated that drug-resistant *Klebsiella pneumoniae* infection significantly decreased A549 cell viability (**Figure 1A**) and promoted cell apoptosis (**Figure 1B**, **1C**). The levels of inflammatory cytokines including TNF-α (**Figure 1D**), IL-6 (**Figure 1E**), and IL-1β (**Figure 1F**) were markedly increased after *Klebsiella pneumoniae* infection. However, treatment with Meropenem failed to restore *Klebsiella pneumoniae* infection-induced viability inhibition, apoptosis, and inflammatory response in A549 cells, indicating that this *Klebsiella pneumoniae* strain was resistant to meropenem.

*TRAIL overexpression enhanced the drug resistance of Klebsiella pneumoniae*

To investigate the roles of TRAIL in the drug resistance of *Klebsiella pneumoniae*, TRAIL ov-

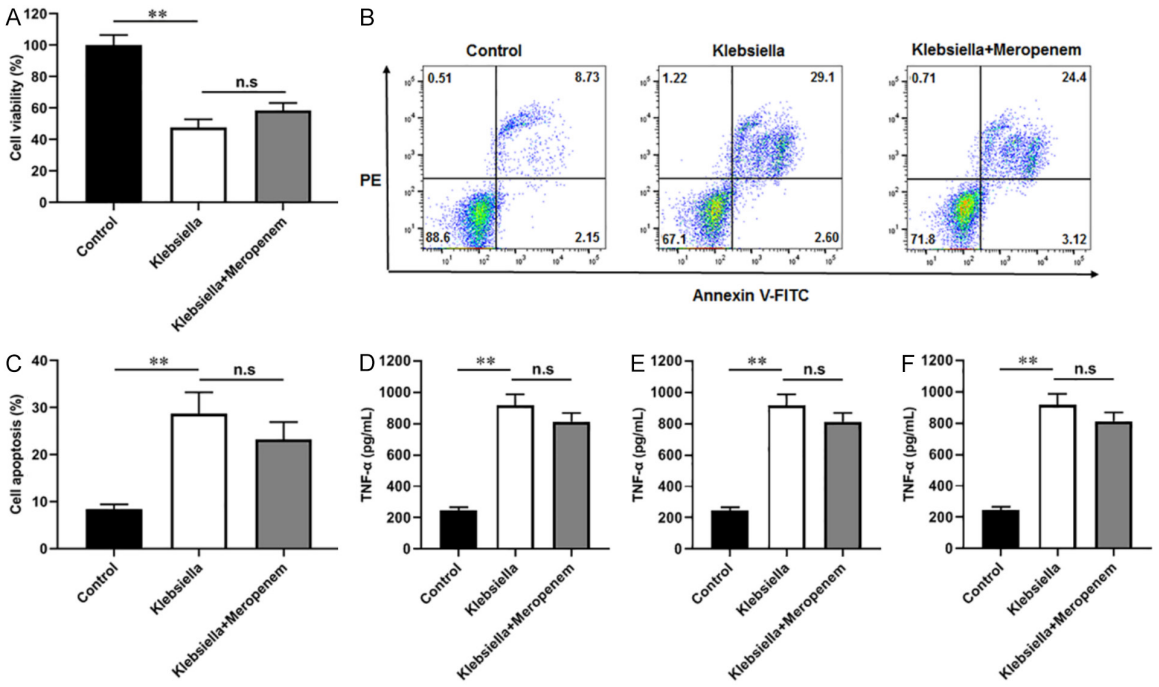


**Figure 1.** The drug-resistant *Klebsiella pneumoniae* infection promoted apoptosis and inflammation in A549 cells. A drug-resistant *Klebsiella pneumoniae*-infected A549 cell model was prepared and treated with meropenem. A: Cell viability was quantified using Cell Counting Kit-8 (CCK-8) assay. B, C: Cell apoptosis was examined using flow cytometry. D-F: Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) levels were assessed using enzyme-linked immunosorbent assay (ELISA) kits. Data are expressed as mean ± standard deviation (SD). $^{**}P < 0.01$.

erexpression plasmids and siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. The transfection efficiencies are shown in **Figure 2A** and **2B**. Transfection of OE-TRAIL increased TRAIL protein levels, while si-TRAIL transfection decreased TRAIL protein. Furthermore, TRAIL overexpression aggravated *Klebsiella pneumoniae* infection-induced viability inhibition and apoptosis in the presence of meropenem, suggesting that TRAIL enhances the drug resistance of *Klebsiella pneumoniae*. In contrast, TRAIL knockdown attenuated *Klebsiella pneumoniae* infection-induced inhibition of viability (**Figure 2C**) and apoptosis (**Figure 2D**, **2E**) under meropenem treatment, indicating that TRAIL inhibition reduces the drug resistance of *Klebsiella pneumoniae*. Additionally, TRAIL overexpression promoted inflammatory cytokine production (**Figure 2F-H**), while TRAIL knockdown showed the opposite results. Our findings collectively reveal the critical effects of TRAIL in regulating the antibiotic resistance of *Klebsiella pneumoniae*.

*TRAIL enhanced the drug resistance of Klebsiella pneumoniae by upregulating armA expression*

To investigate the effects of TRAIL-mediated armA regulation on the drug resistance of *Klebsiella pneumoniae*, OE-TRAIL and armA siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells that were subsequently treated with meropenem. We found that transfection of OE-TRAIL increased armA mRNA expression, while transfection of si-armA inhibited armA mRNA expression (**Figure 3A**). Moreover, TRAIL overexpression aggravated *Klebsiella pneumoniae* infection-induced inhibition of viability (**Figure 3B**) and apoptosis (**Figure 3C**, **3D**) under meropenem treatment, while these effects were abrogated by armA inhibition. Furthermore, TRAIL overexpression exacerbated *Klebsiella pneumoniae* infection-induced inflammatory cytokine production (**Figure 3E-G**). However, this effect was eliminated by armA inhibition. These results revealed that TRAIL overexpression enhances the drug resis-

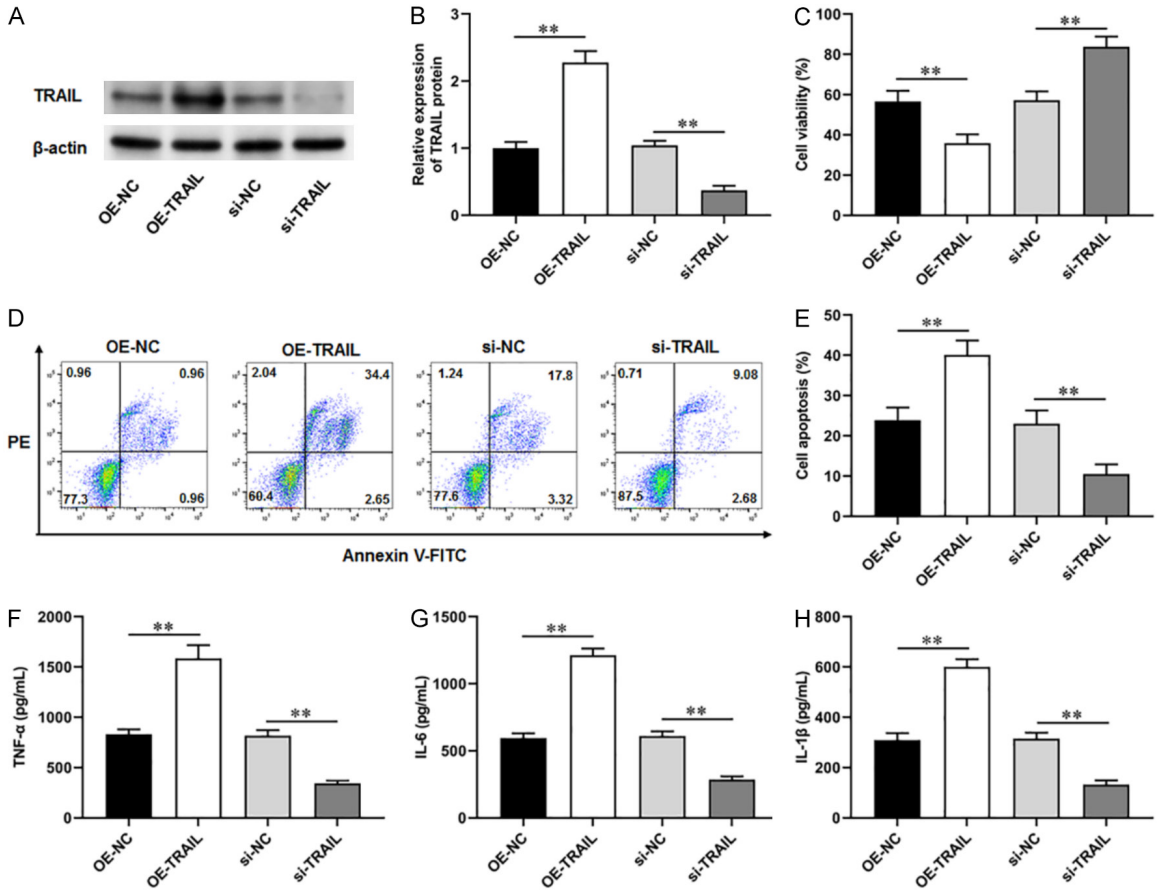


**Figure 2.** Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) overexpression enhanced the drug resistance of *Klebsiella pneumoniae*. TRAIL overexpression plasmids and small interfering RNA (siRNA) were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. A, B: TRAIL protein expression was quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). C: Cell viability was quantified using a CCK-8 assay. D, E: Cell apoptosis was examined using flow cytometry. F-H: TNF-α, IL-6, and IL-1β levels were assessed using ELISA kits. Data are expressed as mean ± SD. $^{**}P < 0.01$.

tance of *Klebsiella pneumoniae* by upregulating armA expression.

*TRAIL overexpression activated the PI3K/AKT/mTOR signaling pathway in Klebsiella pneumoniae-infected A549 cells*

To elucidate TRAIL-mediated regulation of PI3K/AKT/mTOR signaling pathway in the drug resistance of *Klebsiella pneumoniae*, TRAIL overexpression plasmids and siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. Western blot results suggested that TRAIL overexpression increased the phosphorylated levels of PI3K, AKT, and mTOR in *Klebsiella pneumoniae*-infected A549 cells (**Figure 4A-D**). Conversely, TRAIL knockdown suppressed p-PI3K, p-AKT, and p-mTOR levels. Thus, TRAIL has a critical role in activating the PI3K/AKT/mTOR signaling pathway in the antibiotic resistance of *Klebsiella pneumoniae*.

*TRAIL activated the PI3K/AKT/mTOR signaling pathway by upregulating armA expression*

We further explored the effect of TRAIL/armA axis on the PI3K/AKT/mTOR signaling pathway in the drug resistance of *Klebsiella pneumoniae*. OE-TRAIL and armA siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. We found that TRAIL overexpression enhanced p-PI3K, p-AKT, and p-mTOR levels in *Klebsiella pneumoniae*-infected A549 cells, while these effects were eliminated by armA inhibition (**Figure 5A-D**). This showed that the TRAIL/armA axis enhanced the drug resistance of *Klebsiella*

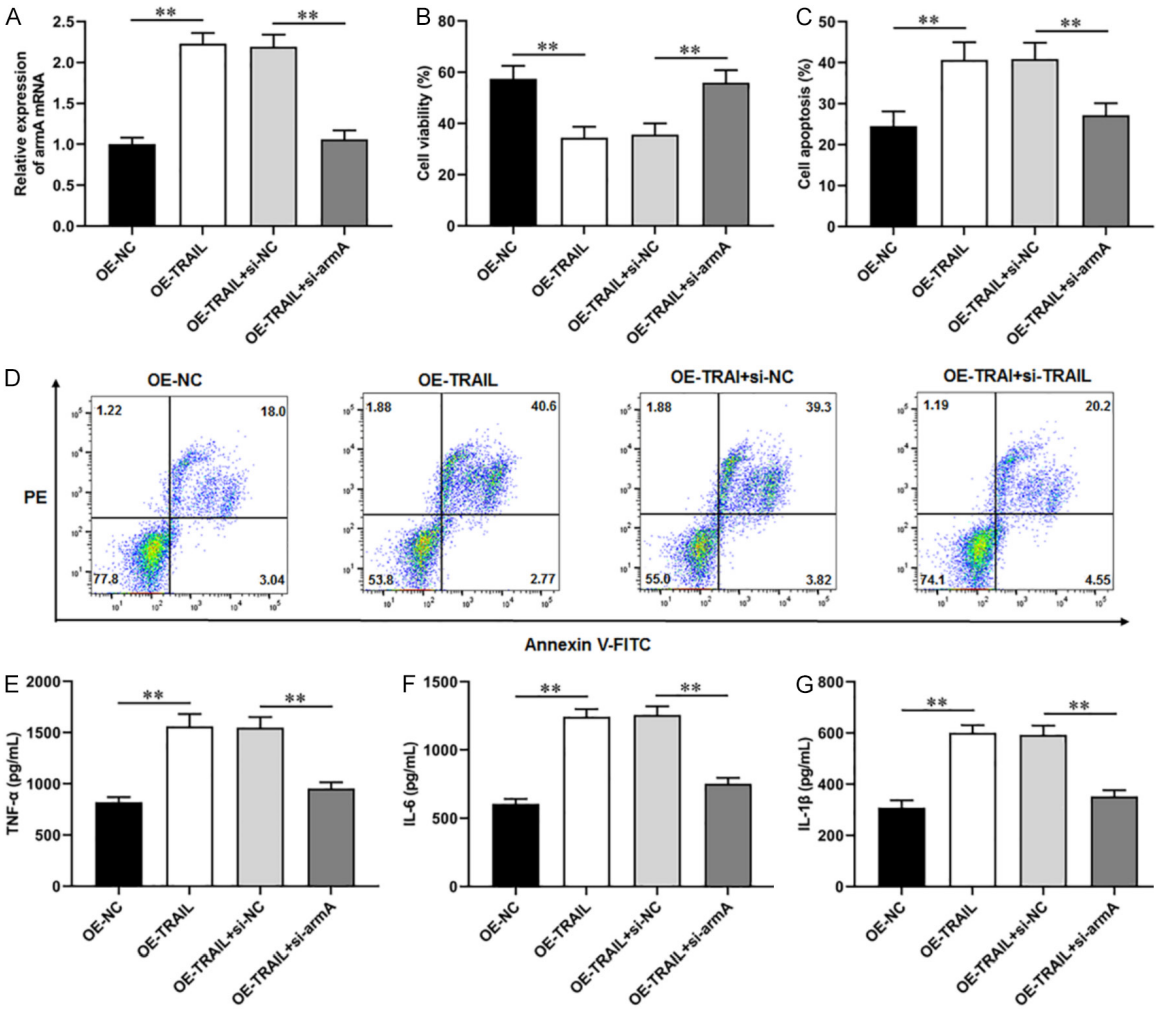


**Figure 3.** TRAIL enhanced the drug resistance of *Klebsiella pneumoniae* by upregulating aminoglycoside resistance methylase (armA) expression. TRAIL overexpression plasmids (OE-TRAIL) and armA siRNA (si-armA) were transfected into *Klebsiella pneumoniae*-infected A549 cells that were subsequently treated with meropenem. A: Relative mRNA expression of armA was quantified using RT-qPCR. B: Cell viability was quantified using CCK-8 assay. C, D: Apoptosis was examined using flow cytometry. E-G: TNF-α, IL-6, and IL-1β levels were assessed using ELISA kits. Data are expressed as mean ± SD. $^{**}P < 0.01$.

*pneumoniae* through activation of the PI3K/AKT/mTOR signaling pathway.

## Discussion

This study provides a comprehensive investigation into the mechanism of the TRAIL-mediated armA methylase gene in conferring drug resistance in *Klebsiella pneumoniae*, with particular emphasis on its regulatory effect on the PI3K/AKT/mTOR signaling pathway. We propose that TRAIL-mediated armA upregulation enhanced the drug resistance of *Klebsiella pneumoniae* by activating the PI3K/AKT/mTOR signaling pathway. These findings provide new insight into the drug resistance mechanisms of *Klebsiella pneumoniae*. We can now critically integrate our findings with those of the existing literature to explain the intricate relationship between TRAIL-mediated armA methylase and drug resistance pathways.

The primary finding of this study was that TRAIL is a crucial modulator regulating the drug resistance of *Klebsiella pneumoniae*. Our experimental results demonstrated that TRAIL knockdown increased cell viability and reduced apoptosis and inflammatory cytokine production in drug-resistant *Klebsiella pneumoniae*-infected A549 cells under meropenem treatment, while TRAIL overexpression exerted opposing effects. These results imply that TRAIL may significantly contribute to the emergence of antibiotic resistance in *Klebsiella pneumoniae*, possibly thr-

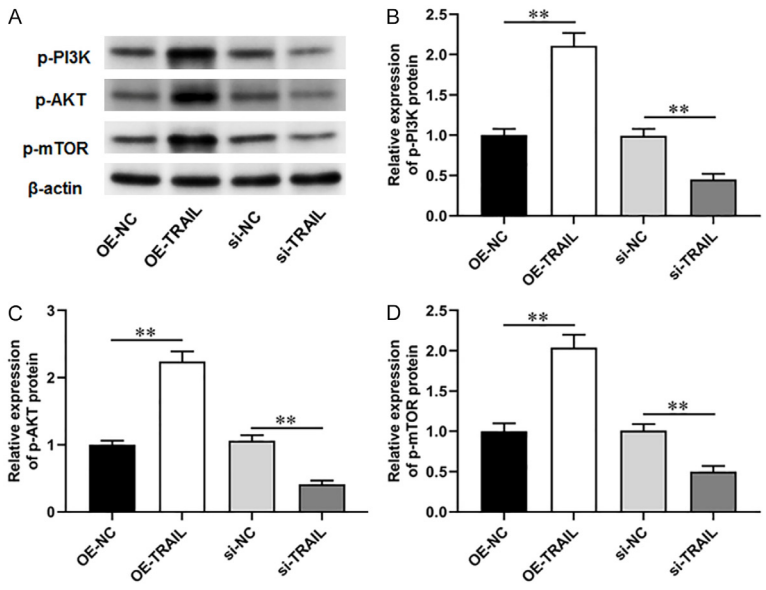


**Figure 4.** TRAIL overexpression activated the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway in *Klebsiella pneumoniae*-infected A549 cells. TRAIL overexpression plasmids and siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. A: Representative images of the protein bands. B-D: The levels of p-PI3K, p-AKT, and p-mTOR were examined using western blots. Data are expressed as mean ± SD. $^{**}P < 0.01$.

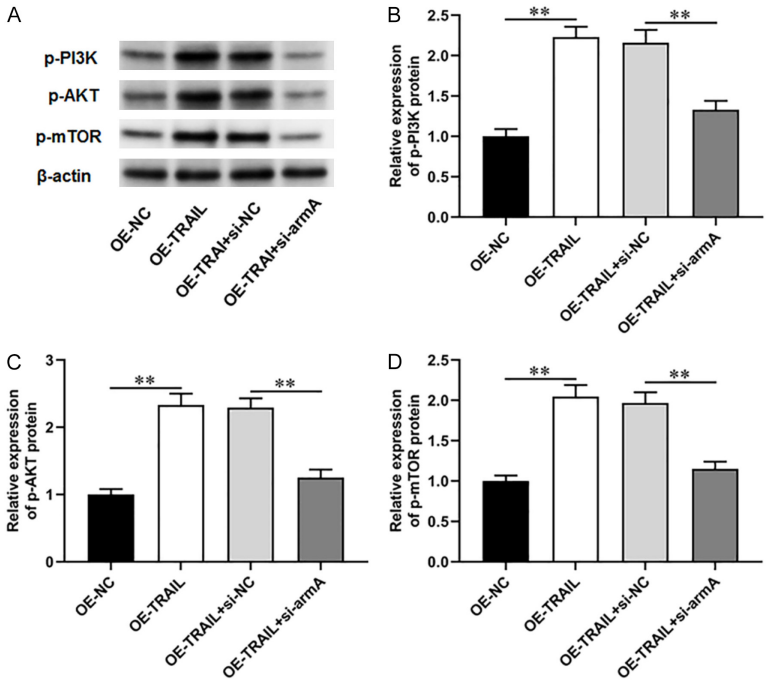


**Figure 5.** TRAIL activated the PI3K/AKT/mTOR signaling pathway by upregulating armA expression. OE-TRAIL and armA siRNA were transfected into *Klebsiella pneumoniae*-infected A549 cells and subsequently treated with meropenem. A: Representative images of the protein bands. B-D: The levels of p-PI3K, p-AKT, and p-mTOR were examined using western blots. Data are expressed as mean ± SD. $^{**}P < 0.01$.

ough mechanisms that modulate host cell responses to bacterial infection. These findings are consistent with prior evidence indicating TRAIL's role in regulating host immune responses to bacterial infections, including those caused by *Klebsiella pneumoniae* [14, 15]. However, despite these advances, the precise mechanisms of TRAIL in regulating bacterial resistance had been under investigated.

Our in-depth study was the first to show that TRAIL-mediated upregulation of the armA methylase gene is a significant feature in drug-resistant *Klebsiella* infections. These findings strongly implicate that armA is critically involved in driving drug resistance in *Klebsiella*. Mechanistically, armA catalyzes the transfer of methyl groups to a specific adenine residue in 16S rRNA, a modification that profoundly influences bacterial protein translation and gene expression [10, 11]. Such epigenetic alterations likely contribute to bacterial survival strategies, particularly in the context of antibiotic resistance. Earlier research had established that the armA methylase gene was a key factor in mediating antibiotic resistance across various bacterial species. For instance, upregulation of armA methylase reduced the binding affinity of between antibiotics and armA, thereby conferring high-level resistance to these antibiotics [22, 23]. In this case, the upregulated armA expression represents as a key drug resistance strategy that helps maintain bacterial survival. Our results are consistent with these findings. We illustrated that TRAIL played a pivotal role in enhancing the

antibiotic resistance of *Klebsiella pneumoniae* by upregulating armA expression. Knockdown of armA inhibited the promoting effect of TRAIL on the drug resistance of *Klebsiella pneumoniae*. These further underscore the contribution of the armA methylase gene to drug resistance in *Klebsiella pneumoniae* and lay the groundwork for future research to elucidate the molecular mechanisms underlying the resistant phenotype.

The upregulation of the armA expression was found to be accompanied by abnormal activation of the PI3K/AKT/mTOR signaling pathway, suggesting a role of this pathway in the development of drug resistance in *Klebsiella pneumoniae* infection. This pathway is a well-characterized cellular signaling network critically implicated in numerous diseases, such as cancer, inflammation, and infections [19-21]. In the context of bacterial infections, its aberrant activation has been linked to enhanced inflammatory and immune responses, which may contribute to bacterial survival and replication [24-26]. Our findings are consistent with previous studies demonstrating that the roles of PI3K/AKT/mTOR pathway in drug resistance of *Klebsiella pneumoniae*. Additionally, our results implicated that TRAIL overexpression activated this pathway, while armA knockdown abrogated TRAIL-mediated abnormal activation of the PI3K/AKT/mTOR signaling pathway. This finding holds the important clinical implication that targeting the armA methylase gene could be a novel strategy to modulate this pathway and combat drug-resistant *Klebsiella pneumoniae*. Nevertheless, additional studies are required to confirm these results and explore specific therapeutic interventions to effectively disrupt this resistance mechanism.

Based on our findings, several therapeutic strategies might be proposed to combat drug-resistant *Klebsiella pneumoniae* infections. First, targeting the TRAIL and armA methylase gene to suppress its expression could be explored as a method to reduce bacterial resistance. Second, modulating the PI3K/AKT/mTOR signaling pathway to inhibit its abnormal activation may enhance the efficacy of existing antibiotics. Pharmacologic inhibitors of this pathway, which have already shown promise in cancer therapy, could be repurposed for this goal. These strategies might also help fight other drug-resistant bacterial infections in addition to *Klebsiella*.

This study has several limitations. First, while we identified a connection between armA and the PI3K/AKT/mTOR pathway, the precise molecular mechanisms underlying this relationship remain unclear. Future studies should investigate the mechanism by which TRAIL regulates armA expression (e.g., transcription factors or epigenetic modifications). Second, all experiments were conducted in an *in vitro* A549 cell model, lacking validation in animal studies. The *in vivo* Klebsiella infection is complex and is influenced by factors such as immune regulation, metabolic differences, and host-pathogen interaction. Thus, future work should employ murine infection models to verify the role of the TRAIL/armA axis in living systems and assess its effect on clinical outcome. Additionally, the study focused on armA and the PI3K/AKT/mTOR pathway, leaving unexplored whether other resistance-related genes (e.g., β-lactamases or efflux pump genes) contribute to TRAIL-mediated resistance.

In conclusion, this study reveals that TRAIL-mediated armA upregulation enhances the drug resistance of *Klebsiella* infection by activating the PI3K/AKT/mTOR signaling pathway. Our discovery provides novel ideas for the treatment of *Klebsiella* infections and is expected to provide new treatment strategies to overcome infections caused by *Klebsiella*. However, further work is needed to validate this mechanism, explore potential treatment strategies, and translate the findings into clinical practice.

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## Disclosure of conflict of interest

None.

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