Antibiotic resistance of *Klebsiella pneumoniae* through β-arrestin recruitment-induced β-lactamase signaling pathway

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Abstract. Overuse and misuse of antibiotics leads to rapid evolution of antibiotic-resistant bacteria and antibiotic resistance genes. *Klebsiella pneumoniae* has become the most common pathogenic bacterium accountable for nosocomial infections due to its high virulence factor and general occurrence of resistance to most antibiotics. The β-lactamase signaling pathway has been suggested to be involved in antibiotic resistance against β-lactams in *Klebsiella pneumoniae*. In the present study, the molecular mechanism of the antibiotic resistance of *Klebsiella pneumoniae* was investigated and the results indicated involvement of the β-arrestin recruitment-induced β-lactamase signaling pathway. Antimicrobial susceptibility of *Klebsiella pneumoniae* was assessed using automated systems and extended-spectrum β-lactamase (ESBL) and β-arrestin expression levels in *Klebsiella pneumoniae* were analyzed by reverse-transcription quantitative PCR. β-lactam resistance in *Klebsiella pneumoniae* was determined using β-lactam agar screening plates. The results demonstrated that β-arrestin recruitment was increased in *Klebsiella pneumoniae* with antibiotic resistance (AR-K.P.) compared with that in the native *Klebsiella pneumoniae* strain (NB-K.P.). Increased production of ESBL was observed in AR-K.P. after treatment with the β-lactam penicillin. Of note, inhibition of β-arrestin recruitment significantly suppressed ESBL expression in AR-K.P. and in addition, genes encoding β-arrestin and ESBL were upregulated in *Klebsiella pneumoniae*. Restoration of endogenous β-arrestin markedly increased antibiotic resistance of *Klebsiella pneumoniae* to β-lactam. Knockdown of endogenous β-arrestin downregulated antibiotic resistance genes and promoted the inhibitory effects of β-lactam antibiotic treatment on *Klebsiella pneumoniae* growth. In conclusion, the present study identified that β-arrestin recruitment was associated with growth and resistance to β-lactams, which suggested that β-arrestin regulating ESBL expression may be a potential target for addressing antibiotic resistance to β-lactams in *Klebsiella pneumoniae*.

Introduction

Antibiotic-resistant bacteria are reported as the greatest threat to human health in the world (1,2). Overuse and misuse of antibiotics contributes to the occurrence of antibiotic resistance genes and the evolution of antibiotic-resistant bacteria according to the discipline of genesis and evolution (3,4). In the past several decades, antibiotic resistance has been accelerated by the indiscriminate application of antibiotics leading to the vital problem of antibiotic resistance and significant public health concerns (5). Antibiotic resistance is a significant challenge for microbiology labs and clinicians (6). Previous studies have suggested that the major driving force in the occurrence of antibiotic-resistant pathogens is the evolution of metabolic function caused by the rapid antibiotic consumption in the world (7,8). Extended-spectrum β-lactamase (ESBL) produced by antibiotic-resistant bacteria is another mechanism underlying the phenomenon of the evolution of antibiotic-resistant bacteria (9,10). Clinically, antibiotic resistance in patients has made treatment approaches for bacterial infections insufficient and resulted in a markedly increased morbidity and mortality (11,12). Therefore, understanding the molecular mechanisms of resistance to antimicrobial agents is imminent to develop novel intervention strategies to improve the survival of patients.

In recent years, antimicrobial resistance and susceptibility of *Klebsiella pneumoniae* have been observed in clinical practice (13). *Klebsiella pneumoniae* has represented an intractable pathogen in pulmonary disease departments, which partly attributed to an increased economic burden, promoted intra-hospital transmission and challenged infection control practices (14). Previous studies have suggested that nosocomial infections of *Klebsiella pneumoniae* frequently erupted due to increased production of ESBL produced by *Klebsiella pneumoniae*, which attributed to multiple mechanisms of drug resistance (15,16). In addition, antibacterial drug susceptibility...
tests of isolated strains of *Klebsiella pneumoniae* revealed that strains have been disseminated with simultaneous resistance to various types of antimicrobial agents, mediated by loss of porins on the cytomembrane, the production of ESBL, carbapenemases and even metallo-β-lactamases to enhance antibiotic resistance (17,18). In these studies, ESBL was reported to have the most important role in the progression of the antimicrobial resistance of *Klebsiella pneumoniae*. Furthermore, clinical research has indicated that ESBL production is frequently upregulated in *Klebsiella pneumoniae* isolated from patients with long-term infection, who are likely to be infected with other bacteria, making it difficult for clinicians to select appropriate antibiotics for treatment (19). However, despite the different types of antibiotics that have been developed, the molecular mechanisms of *Klebsiella pneumoniae* resistance to antimicrobial drugs have remained to be fully elucidated (20).

At present, persistent microbial resistance of *Klebsiella pneumoniae* represents a clinical problem (21). Development of alternative drugs to inhibit anti-microbial resistance for treating hospital-acquired *Klebsiella pneumoniae* infections urgently requires novel approaches. The present study investigated the role of β-arrestin in the β-lactamase signaling pathway in *Klebsiella pneumoniae*. A previous study reported that β-arrestin translocates from the cytosol to the activated receptor and regulates the G protein-coupled receptor signaling pathway (22). In addition, the β-arrestin-mediated pathway is mutually independent by selective regulation of certain ligands in the transmembrane domain (23). Furthermore, studies have suggested that the β-arrestin-mediated signaling pathway may be associated with an unknown accommodation mode (24-26). In agreement with this, the present study found that the β-arrestin-mediated signaling pathway regulates ESBL production in *Klebsiella pneumoniae* with antibiotic resistance.

The present study investigated the correlation between β-arrestin and ESBL production to assess the mechanisms of antimicrobial drug resistance of *Klebsiella pneumoniae*. The results indicated that interference with β-arrestin recruitment or synthesis decreased antimicrobial drug resistance, which may provide an approach for controlling nosocomial infectious, transmission and cross-infection. The association between β-arrestin recruitment, ESBL and mechanisms of antibiotic resistance of *Klebsiella pneumoniae* were studied in *vitro* and in *vivo*. The results revealed that inhibition of the recruitment of β-arrestin inhibited ESBL synthesis and decreased the potential of antimicrobial drug resistance of *Klebsiella pneumoniae*, which validated the role of the β-arrestin-induced β-lactamase signaling pathway in the β-lactam resistance of *Klebsiella pneumoniae*.

**Materials and methods**

**Bacterial culture and reagents.** A native *Klebsiella pneumoniae* strain (NB-K.P.) was purchased from the American Type Culture Collection (Manassas, VA, USA). The antibiotics-resistant strain AR-K.P. was isolated from a 56-year male patient with pneumonia who suffered from pneumonia for ~30 years. *Klebsiella pneumoniae* cells were grown in Lysogeny broth (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C humidified atmosphere containing 5% CO₂ for 24 h.

**Growth potential assay.** The growth potential was assessed according to the protocol of a previous study (27). The *Klebsiella pneumoniae* cells were cultured in medium containing 10 mg/ml penicillin for 24 h. The number of *Klebsiella pneumoniae* colonies was calculated on agar plates.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility tests of *Klebsiella pneumoniae* were performed using the disk diffusion method, according to the Clinical and Laboratory Standards Institute recommendations (28). The final results were obtained according to the respective standards for antimicrobial susceptibility testing using Mueller-Hinton Broth medium in agar plates (Merck KGaA; Darmstadt, Germany).

**β-arrestin activity.** *Klebsiella pneumoniae* clones were seeded in Mueller–Hinton Broth medium in agar plates for 48 h at 37°C. Following the addition of the Flash detection reagent (10 mg/ml; Discoverx; Birmingham, UK), β-arrestin-luciferase signal was read using a fluorescence imaging plate reader (FLIPR Tetra; Molecular Devices, LLC; Sunnyvale, CA, USA).

**β-arrestin endogenous or ESBL expression.** *Klebsiella pneumoniae* cells were cultured in Mueller-Hinton Broth medium in agar plates (Darmstadt, Germany) for 24 h at 37°C. *Klebsiella pneumoniae* cells were prepared and used to make competent cells according to previous report (30). β-arrestin or ESBL gene was cloned and inserted into pET-28a plasmids using a transformation method described previously (31). Cells were checked by colony PCR and confirmed by sequence analysis of the PCR products as described previously (32).

**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was obtained from *Klebsiella pneumoniae* cells by using the RNeasy Mini kit (Qiagen, Hilden, Germany). Expression of β-arrestin and ESBL in cells was measured by applying an RT-qPCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) (33). All of the forward and reverse primers were synthesized by Thermo Fisher Scientific, Inc. (Table I). Relative mRNA expression changes were calculated by the 2^-ΔΔCT^ method (34). The results are expressed as the fold of the β-actin control.

**Animal study.** The study was approved by the Ethics Committee of Tianjin First Center Hospital (Tianjin, China). A total of 60 female BALB/c nude mice (age, 6 weeks;
body weight, 30-35 g) were purchased from Charles River Laboratories GmbH (Sulzfeld, Germany). All animals were fed under pathogen-free conditions. Mice were maintained under a 12-h light/dark cycle with free access to food and water. The mice were randomly divided into four groups, into which different types of Klebsiella pneumoniae cells (AR-K.P., AR-K.P.-β-arrestin-knockdown, NB-K.P. or NB-K.P.-arrestin-knockdown) were injected into the vena caudalis (10⁸ infective particles per mouse). The onset of illness was observed for each mouse in every group. All mice received penicillin (0.3 mg/kg; Sigma-Aldrich; Merck KGaA) treatment on day 24. The penicillin treatment was continued for 15 days with administration once daily. On day 40, lungs were isolated and used for protein analysis by immunohistochemical staining. The efficacy of β-arrestin knockdown in the progression of pneumonia was determined by PSI score of pneumonia mice (35).

Immunofluorescence. Klebsiella pneumoniae cells or lung tissues were fixed with formaldehyde solution (10%) and processed according to standard procedures. Rehydrated slides or cells were incubated with primary antibodies: β-arrestin (1:200 dilution; 30036; Cell Signaling Technology, Inc., Danvers, MA, USA), ESBL (1:200 dilution; 90122; Cell Signaling Technology, Inc.) and β-actin (1:1,000 dilution; 91763; Cell Signaling Technology, Inc.) and Blal (1:1,000 dilution; 97364; Cell Signaling Technology, Inc.), BlaR1 (1:1,000 dilution; 91763; Cell Signaling Technology, Inc.) and ESBL (1:200 dilution; 90122; Cell Signaling Technology, Inc.) for 12 h at 4˚C. Lung sections were then incubated with immunoperoxidase by means of a 3,3′-diaminobenzidine kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. The sections were examined under a light microscope at a magnification of x400.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Unpaired data were analyzed by Student’s t-test. Comparisons of data between multiple groups were performed by analysis of variance. All data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 and was considered to indicate a statistically significant difference.

Results

β-arrestin and ESBL gene expression and antimicrobial drug resistance of Klebsiella pneumoniae. In order to assess the drug resistance of Klebsiella pneumoniae to β-lactams, β-arrestin and ESBL gene expression were evaluated in AR-K.P. As presented in Fig. 1A and B, β-arrestin and ESBL expression levels were downregulated in AR-K.P. compared to those in NB-K.P. As displayed in Fig. 1C, AR-K.P. was resistant to the antimicrobial drugs clindamycin, erythromycin, linezolid and penicillin. In addition, the location of β-arrestin and ESBL proteins in AR-K.P. was identified. The results indicated that β-arrestin was located in the intracellular compartment and that ESBL was expressed in the cytomembrane (Fig. 1D). These results suggested that Klebsiella pneumoniae is resistant to antimicrobial drugs and that β-arrestin and ESBL gene expression levels were upregulated in AR-K.P.

Roles of β-arrestin and ESBL in Klebsiella pneumoniae growth and anti-microbial drug resistance. In order to assess the role β-arrestin and ESBL in Klebsiella pneumoniae growth, the activities of β-arrestin and ESBL in Klebsiella pneumoniae were first evaluated. As presented in Fig. 2A, the β-arrestin-luciferase signal was higher in AR-K.P. compared with that in NB-K.P. The growth of Klebsiella pneumoniae in the presence of penicillin (10 mg/l) was prompted after endogenous β-arrestin expression (Fig. 2B). As displayed in Fig. 2C, ESBL expressed in AR-K.P. more efficiently hydrolyzed β-lactam compared with that in NB-K.P. Of note, restoration of ESBL in Klebsiella pneumoniae contributed to the capacity of antimicrobial drug resistance, resulting in the promotion Klebsiella pneumoniae growth in penicillin-containing medium (Fig. 2D). These results indicated that β-arrestin and ESBL enhanced antimicrobial drug resistance and promoted the growth of Klebsiella pneumoniae.
Knockdown of β-arrestin decreases ESBL expression and growth of Klebsiella pneumoniae. To identify the association between β-arrestin, ESBL and the antimicrobial drugs resistances of Klebsiella pneumoniae, the β-arrestin-mediated signaling pathway was analyzed. As presented in Fig. 3A, β-arrestin stimulated ESBL production in Klebsiella pneumoniae and in a dose-dependent manner. The increasing production of ESBL allowed Klebsiella pneumoniae to survive in an environment containing penicillin antibiotics (Fig. 3B). Of note, β-arrestin knockdown led to downregulation of ESBL.
expression and inhibited the growth of *Klebsiella pneumoniae* (Fig. 3C and D). β-arrestin knockdown also attenuated ESBL signal intensity on the surface of *Klebsiella pneumoniae* (Fig. 3E). Collectively, these findings suggested that β-arrestin knockdown induced the downregulation of ESBL, which attenuated the antimicrobial drug resistance of AR-K.P.

Knockdown of β-arrestin decreases the virulence and antimicrobial drug resistance of *Klebsiella pneumoniae* in vivo. To investigate the inhibitory effects of β-arrestin knockdown on the biotic resistance capacity of *Klebsiella pneumoniae*, mice were infected with *Klebsiella pneumoniae* with or without β-arrestin knockdown and treated with penicillin. In mice infected with *Klebsiella pneumoniae* with β-arrestin knockdown, the onset of pneumonia was delayed (Fig. 4A). Penicillin treatment was also found to efficiently inhibit the proliferation of *Klebsiella pneumoniae* with β-arrestin knockdown, resulting in rapid recovery of pneumonia mice, as indicated by the decline in pneumonia score (Fig. 4B). Immunohistochemistry revealed that the *Klebsiella pneumoniae* cells with β-arrestin knockdown were almost eradicated after a 15-day penicillin treatment compared to that in the control groups (Fig. 4C). Of note, the expression levels of two important members of the β-lactamase signal pathway in *Klebsiella pneumoniae*, β-lactamase repressor (BlaR)1 and BlaI, were markedly decreased after penicillin treatment on day 7 (Fig. 4D and E). In addition, the results demonstrated that β-arrestin and ESBL expression were significantly downregulated in the lungs of mice infected with *Klebsiella pneumoniae* with β-arrestin knockdown (Fig. 4F and G). Taken together, these findings suggested that knockdown of β-arrestin decreased the virulence and antimicrobial drug resistance of *Klebsiella pneumoniae* in vivo.

**Discussion**

At present, treatment with β-lactam-based antibiotics is the most common therapeutic strategy for *Klebsiella pneumoniae* infection in the clinic (36). However, overuse and misuse of antibiotics leads to rapid evolution of antibiotic-resistant *Klebsiella pneumoniae* strains. In addition, clinical evidence has suggested that *Klebsiella pneumoniae* has become the most common pathogenic bacterium causing nosocomial infectious due to the high virulence factors and general occurrence of resistance to most antibiotics (37,38). The association between antimicrobial drug resistance and biofilm formation along with ESBL lactamase produced in *Escherichia coli* has been assessed in a previous study (39). In line with this, the present study indicated that antibiotic resistance of *Klebsiella pneumoniae* depended on the expression of ESBL, which efficiently hydrolyzed penicillin present in the medium. The results demonstrated that β-arrestin recruitment significantly regulated ESBL expression, resulting in a marked improvement of antibiotic resistance of *Klebsiella pneumoniae*.
These findings suggested that β-arrestin may be a potential target to address the antibiotic resistance of *Klebsiella pneumoniae*.

Based on previous studies, the increasing antibiotic resistance of gram-negative bacilli enhances the rate of associated morbidities and mortalities worldwide (40,41). *Klebsiella pneumoniae* is a gram-negative bacterium and an epidemiological study based on clinical data has revealed that antibiotic resistance of *Klebsiella pneumoniae* is increasing (42). However, antibiotic treatment is the most common therapeutic regimen...
for pulmonary infections with *Klebsiella pneumoniae* in patients with pneumonia (43). Clinical experience has demonstrated that antibiotic resistance of *Klebsiella pneumoniae* has caused great problems in the treatment of pneumonia, as only few antibiotic drugs may be applied to patients infected with *Klebsiella pneumoniae* (44). Among these antibiotic drugs, β-lactam antibiotics exhibit a higher efficiency in killing *Klebsiella pneumoniae*. However, in recent years, drug resistance of *Klebsiella pneumoniae* has led to insufficient efficacy of β-lactam antibiotics (45). A study has indicated that extended-spectrum β-lactamase produced by *Klebsiella pneumoniae* may be associated with antibiotic resistance (46). The mechanism of β-lactamase hydrolyzing β-lactam antibiotics has been demonstrated in *Klebsiella pneumoniae* (47). A previous study has identified evolutionarily conserved genetic associations between β-lactamases and antibiotic-producing bacteria (48). In addition, antibiotic resistance genes originating from antibiotic-producing microorganisms may be integrated into the genome of other pathogens through transduction and/or transformation (49,50).

Theoretically, simultaneous and long-term analysis of *Klebsiella pneumoniae* resistance to various antimicrobials may contribute to an enhanced knowledge for improving current and future perspectives regarding the therapeutic use of these drugs (51). In a previous study, β-arrestin recruitment was found to be closely associated with β-lactamase enzyme fragments and with antibiotic resistance of *Klebsiella pneumoniae* (52). The present study revealed that β-arrestin knockdown regulated ESBL on the surface of *Klebsiella pneumoniae*, which decreased antibiotic resistance in vivo. Antibiotic-resistant bacteria and antibiotic resistance genes can be passed on between different bacteria (7), which supports that clinical resistance is intimately correlated with environmental resistance (8,9). The present study found that β-arrestin may be a potential target for inhibiting the antibiotic resistance of *Klebsiella pneumoniae*.

In conclusion, despite the trend of the antimicrobial susceptibility of *Klebsiella pneumoniae* continuously decreasing due to the emergence of antibiotic resistance, the molecular mechanisms of this resistance have remained to be fully elucidated. The present study demonstrated that regulation of β-arrestin modifies ESBL expression in *Klebsiella pneumoniae*. Importantly, knockdown of β-arrestin gene expression reduced the antibiotic-resistant features of *Klebsiella pneumoniae*. In line with previously reported pre-clinical findings (53), the present study revealed that following β-arrestin knockdown, the mean number of *Klebsiella pneumoniae* cells was significantly decreased in the presence of penicillin, and that the sensitivity to antibiotics was restored in vivo. Taken together, the molecular mechanisms of *Klebsiella pneumoniae* resistance to antibiotics proceed through the β-arrestin recruitment-induced ESBL signaling pathway, suggesting that β-arrestin may be a potential target to address antibiotic resistance of *Klebsiella pneumoniae*.

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