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Identification of an anti-Gram-negative bacteria agent disrupting the interaction between lipopolysaccharide transporters LptA and LptC

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ABSTRACT

Introduction: The emergence of drug-resistant Gram-negative bacteria is a serious clinical problem that causes increased morbidity and mortality. However, the slow discovery of new antibiotics is unable to meet the need for treating bacterial infections caused by drug-resistant strains. Lipopolysaccharide (LPS) is synthesized in the cytoplasm and transported to the cell envelope by the LPS transport (Lpt) system. LptA and LptC form a complex that transports LPS from the inner membrane to the outer membrane.
Methods: This study performed a screen for agents that disrupt the transport of LPS in Gram-negative bacteria *Escherichia coli*. It established a yeast two-hybrid system to detect LptA-LptC interaction and used this system to identify a compound, IMB-881, that blocks this interaction and shows antibacterial activity.
Results: This study demonstrated that the IMB-881 compound specifically binds to LptA to disrupt LptA-LptC interaction using surface plasmon resonance assay. Overproduction of LptA protein but not that of LptC lowered the antibacterial activity of IMB-881. Strikingly, *Escherichia coli* cells accumulated 'extra' membrane material in the periplasm and exhibited filament morphology after treatment with IMB-881.
Conclusion: This study successfully identified, by using a yeast two-hybrid system, an antibacterial agent that likely blocks LPS transport in Gram-negative bacteria.

1. Introduction

The emergence of drug-resistant Gram-negative bacterial strains has caused significant clinical problems because the available antibiotics for treating these infections are limited [1,2]. The discovery of new antibiotics against Gram-negative bacteria has slowed down in recent years and cannot keep pace with the emergence of drug-resistant bacteria [3,4]. Therefore, it is important to identify new antimicrobial compounds against Gram-negative bacteria with novel targets [5]. The envelope of Gram-negative bacteria, such as *Escherichia coli*, consists of an inner membrane (IM), peptidoglycan, outer membrane (OM), and periplasmic space between IM and OM [6]. As a peculiar component of the outer leaflet of the OM, lipopolysaccharide (LPS) contributes to the OM integrity and functions as the permeability barrier, which enables bacteria to survive in harsh environments and to exclude some antibiotics [7].

The LPS consists of lipid A, an O-antigen polysaccharide chain, and a polysaccharide outer core. Since the LPS is synthesized in the cytoplasm, it must be exported to the cell surface by crossing two membranes [8–10]. The LPS transport (Lpt) system is responsible for the transport of LPS. The lipopolysaccharide transport (Lpt) system consists of seven proteins – LptA, B, C, D, E, F, and G – which can be divided into three functional groups: the IM complex LptBFGC, the periplasmic protein LptA, and the OM complex LptDE [11]. As an ATP-binding cassette (ABC) transporter, the LptBFG complex is responsible for extracting LPS from the IM [12]. LptC binds to the LptBFG complex at the outer leaflet of IM and serves as the docking site for LptA, which transports LPS across the periplasmic space toward the OM [13–15]. Once LPS

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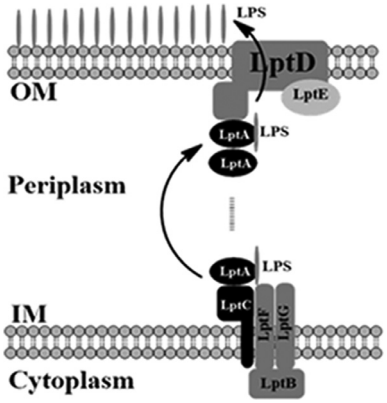

Fig. 1. A diagram of the lipopolysaccharide transport system.

is extracted from the IM by the LptBFG complex, LptA accepts LPS through its interaction with LptC [14,16]. The subsequent interaction between LptA and OM-localized LptD targets LPS to the OM, where the LptDE complex translocates LPS across the OM for the final stage of LPS assembly at the cell surface (Fig. 1) [17–19].

Due to the essential role of LPS in the envelope integrity of Gram-negative bacterial pathogens, LPS biosynthesis is an attractive target for novel antibiotics. In recent years, some potent drug candidates have been identified that inhibit the activity of enzymes responsible for the biosynthesis of lipid A, which is an essential component of LPS [20–24]. Because the Lpt, system is necessary for LPS transport and assembly at the OM, this system should be an ideal target for new antibiotics that inhibit LPS assembly at the OM of Gram-negative bacteria.

The current study developed a screening system based on the interaction between *Escherichia coli* (*E. coli*) proteins LptA and LptC. First, we adopted the yeast two-hybrid (Y2H) system to confirm LptA-LptC interaction and used this system to identify a compound, IMB-881, that blocked LptA-LptC interaction and inhibited the growth of *E. coli*. Moreover, surface plasmon resonance (SPR) assay confirmed that compound IMB-881 inhibited LptA-LptC interaction by specifically binding to LptA protein. In line with this observation, overproduction of LptA, but not of LptC, lowered the antibacterial activity of IMB-881. Moreover, IMB-881 induced abnormal membrane morphology and caused accumulation of 'extra' membrane material in the periplasm. Strikingly, *E. coli* cells treated with this compound exhibited pronounced filament morphology, which could be a result of abnormal cell envelope structure. Therefore, using the Y2H assay, a new antibacterial agent that disrupts the LptA-LptC interaction in Gram-negative bacteria was successfully identified, which validates the LPS transport pathway as a feasible target for new antibacterials.

2. Materials and Methods

2.1. Yeast two-hybrid assay

The construction of the Gal4 Two-Hybrid system (Clontech) was performed as previously described [25]. Briefly, the DNA fragments that encode full-length LptA and LptC proteins were amplified by PCR from the genome of *E. coli* ATCC 25922. The primers used were: *LptA* forward primer, 5'-CATATGAAATTCAAAACAAAC AAACTC-3'; *LptA* reverse primer, 5'-GGATCCTTAATTACCCTTCTTCTGT-3'; *LptC* forward primer, 5'-CTCATATGAGTAAAGCCAGACGTTG-3'; and *LptC* reverse primer, 5'-GGATCCT -TAATTACCCTTCTTCTGT-3'. The PCR fragments were inserted into pGBKT (activation domain, AD) and pGADT7 (DNA binding domain, BD) to generate plasmids pAD-LptA or pBD-LptC. The control plasmids pBD-53, pBD-lam, and pAD-T were obtained from Clontech. The two plasmids pAD-LptA and pBD-LptC were co-transformed into yeast AH109 cells by LiAc protocol to obtain AH109 (pAD-LptA + pBD-LptC). Similarly, AH109 (pBD-LptC + pAD) and AH109 (pAD-LptA + pBD) were obtained. Strains AH109 (pBD-Lam + pAD-T) and AH109 (pBD-P53 + pAD-T) were used as negative control and positive control, respectively. All these strains were grown on synthetic dropout (SD) plates lacking leucine and tryptophan (SD/–Leu/–Trp, clontech) and incubated at 30°C for 2–3 days. The transformants were further inoculated onto SD plates lacking leucine, tryptophan, adenine and histidine (SD/–Leu/–Trp/-Ade/-His, clontech), which were incubated at 30°C for 3–4 days to examine the growth. The positive clones were further assessed using the β-galactosidase assay kit (Thermo).

2.2. Compound library screen

The compound library is a combination of synthetic (from Enamine) and natural products from the Institute of Medicinal Biotechnology. The screening assays were performed as described [26]. In brief, cells (OD_{600}=0.8) of AH109 (pAD-LptA + pBD-LptC) and AH109 (pAD-T + pBD-53) were diluted 100-fold in SD – Leu/–Trp/–Ade/–His dropout medium, but AH109 yeast cells without plasmids were diluted in a yeast peptone dextrose (YPD)-rich medium. The final concentration of compound was 10 μg/mL in 0.1% Dimethyl sulfoxide (DMSO). A total of 5000 compounds were used for this screen. The growth inhibition of the yeast cells was assessed after 36–48 h incubation at 30°C.

2.3. Expression and purification of recombinant LptA and LptC proteins

Full-length LptA and LptC encoding genes were inserted into a pET16b expression vector between the NdeI and BamHI restriction sites. *E. coli* BL21 (DE3) was used to express His-tagged LptA and LptC. The expression of LptA and LptC was induced by 0.5 mM IPTG overnight at 20°C and determined by 12% SDS-PAGE followed by Coomassie Blue staining. Cells were harvested and disrupted by Constant Systems Limited. After centrifugation for 60 min (12 000 × g), the supernatants were loaded onto 5-mL columns of His-Trap HP (GE Healthcare) and then LptA and LptC proteins were eluted using a linear gradient elution buffer. Finally, the purified protein samples were desalted using Amicon Ultra-15 Centrifugal Filter Units from Millpore (Maryland, USA). Protein concentrations were determined using the Bradford method. The purified proteins were confirmed by western blotting using anti-His antibody.

2.4. Surface plasmon resonance (SPR)

The interaction between LptA and LptC was analyzed using BiacoreT200 (GE Healthcare, Sweden). LptA proteins were immobilized on a CM5 sensor chip using an amine coupling kit (GE Healthcare). LptC proteins were passed over the CM5 sensor chip with HBS-EP running buffer. The interaction between proteins and compound IMB-881 was measured using an NTA sensor chip, which was pre-primed by injection of 500 mM nickel chloride in PBS-P running buffer with 5% DMSO. LptA (10 μg/mL) was flowed over the surface of the chip to facilitate binding. Compound IMB-881 was diluted in running buffer and passed over the chip. The interaction of LptC and compound IMB-881 was detected as described for LptA. To obtain inhibition sensor of compound IMB-881, LptC was immobilized on the CM5 sensor chip and 10 μg/mL LptA with 12.5 nM compound in HBS-EP with 5% DMSO was passed over the CM5 sensor chip. Both association (Ka) and dissociation (Kd) values were determined with Biacore T200 Software v2.0.

2.5. The inhibition of Escherichia coli growth by compound IMB-881

Growth inhibition by IMB-881 to the *E. coli* strain ATCC 25922 and clinical isolates was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The final concentrations of IMB-881 ranged 1.5625–100 μg/mL. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that inhibits cell growth.

2.6. Testing the mode of action: Bacteriostatic vs. bactericidal mode

Escherichia coli ATCC 25922 was cultured in LB medium containing IMB-881 (0–4 × MIC). An initial inoculum of around 1×10^7 CFU/mL was used in this experiment. Bacteria were collected at different time points, serially diluted, and spread onto LB plates. After incubation at 37°C for 24 h, the number of colonies was counted.

2.7. Light microscopy and transmission electron microscopy

Escherichia coli ATCC 25922 was diluted to 5×10^6 CFU/mL in LB medium containing IMB-881 (from 6.25–25 μg/mL). Cells were collected every hour and spread onto a slide. Some of the samples were stained by crystal violet, and images were obtained using a ZEISS A-Plan 100 × /1.25 oil immersion objective and analyzed by AxioVision Rel.4.8. Other samples were fixed with 2.5% glutaraldehyde, further fixed with 1% osmium tetroxide in sodium cacodylate buffer for 2 h, and dehydrated with ethanol in serially increased concentrations. The samples were then infiltrated with araldite resin. Embedded samples were sectioned and observed at 80 kV on a JEM-1400 transmission electron microscopy (TEM) from Japan Electronics Co. Ltd (JEOL) [27].

2.8. Isolation of periplasmic fractions for the analysis of lipopolysaccharide levels

Escherichia coli ATCC 25922 was treated with DMSO or 6.25 μg/mL IMB-881 until OD_{600} reached 0.6. Cells were converted to spheroplasts, as previously described [28]. The spheroplasts and residual membranes were removed by ultracentrifugation at 38,000 × g for 30 min at 4°C. The LPS was detected by western blotting using anti-LPS monoclonal antibody at 1:3000 (HyCult Biotechnology, Netherlands).

3. Results

3.1. Interaction between LptA and LptC proteins from Escherichia coli in yeast two-hybrid assay

It has been previously shown that the interaction between LptA and LPS is mediated by LptC, and the interaction between LptA and LptC is required for this replacement [16,29,30] (Fig. 1). In this study, yeast two-hybrid assay (Y2H) was used to confirm the interaction between the LptA and LptC, in order to identify compounds that specifically disrupt this interaction (Fig. 2. A). For this purpose, the plasmids pAD-LptA, pAD-LptC, pBD-LptA and pBD-LptC were constructed. A pair of plasmids of pAD-LptA and pBD-LptC, or pAD-LptC and pBD-LptA was introduced to the AH109 yeast strain. The interaction between LptA and LptC is expected to bring the DNA binding domain (BD) and activating domain (AD) of transcription factor Gal4 together, and activate the transcription of three reporter genes *ADE2, HIS3*, and *LacZ* in AH109 yeast cells. Thus, LptA-LptC interaction can be determined based on the growth of yeast cells on plates lacking adenine and histidine (synthetic defined (SD) –Ade/−His), as well as the strong β-gal activity using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the LacZ substrate. AH109 yeast cells with plasmids pAD-T and pBD-53, which express proteins p53 and SV40-T, were used as a positive control, as these two proteins interact with each other. AH109 cells with pAD-T and pBD-lam (human lamin C) were used as a negative control.

All AH109 transformants could grow on SD –Leu/–Trp dropout plates, indicating that the plasmids were successfully transformed. The positive control and AH109 cells with pAD-LptA and pBD-LptC grew well on an SD –Ade/−His dropout plate and showed strong β-gal activity, indicating the interaction between LptA and LptC. Negative AH109 control cells and cells expressing LptA or LptC alone did not grow on SD –Ade/−His dropout plates, excluding the possibility of self-activation (Fig. 2.B and C). Surprisingly, AH109 with pAD-LptC and pBD-LptA plasmids did not show growth on the dropout plates. Due to the essential N-terminal domain of LptA being responsible for the interaction with LptC [16,29], it was speculate that the fusion of the DNA binding domain of Gal4 at the N terminus of LptA protein may affect its interaction with LptC. The current study also validated the expression of LptA and LptC proteins in yeast cells using western blot assay (Fig. 2.D). Therefore, the interaction between two *E. coli* proteins LptA and LptC can be successfully detected with the Y2H system.

3.2. Compound IMB-881 inhibits LptA-LptC interaction using Y2H

To identify compounds that can disrupt the interaction between LptA and LptC, the growth of AH109 cells with pAD-LptA and pBD-LptC plasmids were assessed in the presence of compounds at 10 μg/mL. Because the compounds that block Gal4 expression or show antifungal activity also inhibit the growth of AH109 (pAD-LptA + pBD-LptC), AH109 (pAD-T + pBD-53) was used as a positive control. The compounds that show specific growth inhibition for AH109 (pAD-LptA + pBD-LptC), but not for AH109 (pAD-T + pBD-53), were selected. From this screen, five compounds were identified, including IMB-881, which likely inhibits LptA-LptC interaction based on the Y2H assay (Fig. 3.A). The structure of compound IMB-881 is shown in Fig. 3.B. To further confirm that these compounds selectively block LptA–LptC interaction, liquid β-gal assay was also performed. As expected, yeast strains AH109 (pAD-LptA + pBD-LptC) showed dose-dependent inhibition of β-gal activity by IMB-881. The β-gal activity of strain AH109 (pAD-T + pBD-53) was also inhibited by this compound, but to a lesser extent (Fig. 3.C). These results indicate that compound IMB-881 likely disrupts LptA-LptC interaction. Compared to IMB-881, other compounds are less efficient in blocking LptA-LptC interaction; therefore, IMB-881 was the focus of this research.

3.3. Compound IMB-881 blocks LptA-LptC interaction by binding to LptA

The disruption of LptA-LptC interaction by IMB-881 was further analyzed using SPR, which is widely used for quantitatively measuring intermolecular interactions in real time [31]. For this purpose, the study first expressed N-terminal 10-His-tagged LptA and LptC proteins in *E. coli*, and purified proteins were used to examine their interaction by SPR. The CM5 sensor chip was coated with 500RU LptA and then exposed to LptC at various concentrations. LptC could bind to LptA, as evidenced by a measurable change in response units, and this change was dose-dependent (Fig. 4.A). The kinetic parameter KD value was $4.706E^{-7}$. To determine whether compound IMB-881 binds to LptA or LptC, or both, the NTA sensor chip was coated with LptA or LptC and then exposed to IMB-881 at various concentrations. The sensorgrams obtained from the Biacore analysis demonstrated that IMB-881 showed a strong binding to LptA, but not to LptC (Fig. 4.B and C), suggesting that LptA is the target of this compound. It was noticed that there was a unit change of the SPR results after the sensor chip was switched from

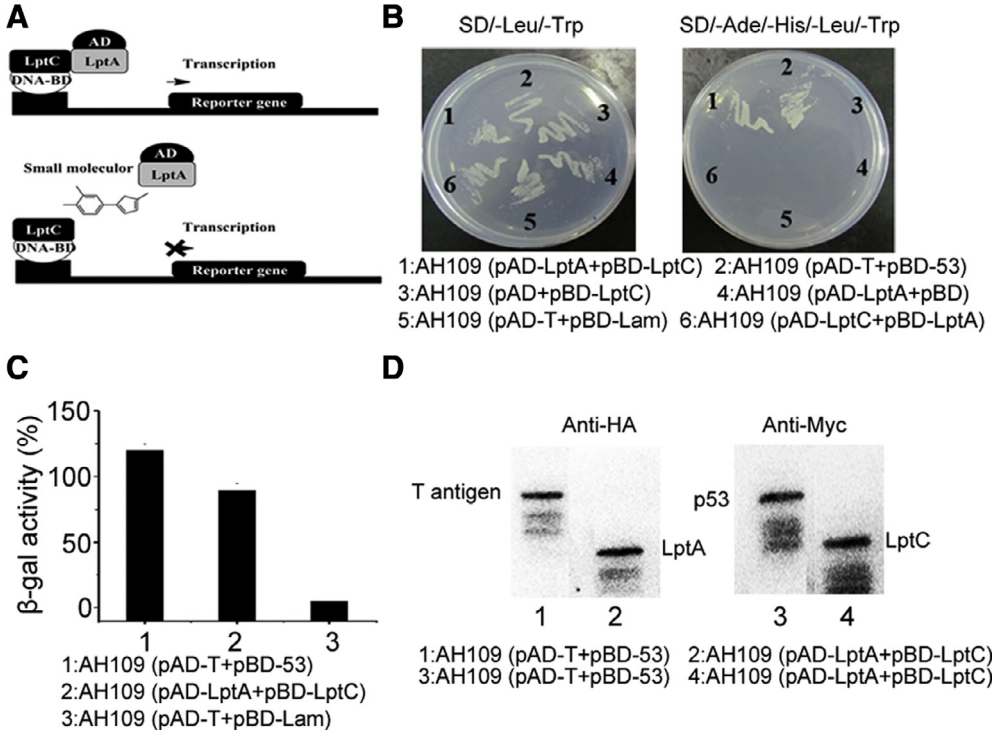

Fig. 2. The establishment of a yeast two-hybrid assay to detect LptA–LptC protein interaction.
(A) The strategy for high throughput screening using the yeast two-hybrid system. The interaction between LptA and LptC proteins reconstitutes the function of transcription factor Gal4 and results in the expression of the reporter genes *ADE2*, *HIS3*, and LacZ. The compounds that disrupt LptA–LptC interaction prevent expression of these reporter genes.
(B) The growth of yeast cells expressing various combinations of BD (DNA binding domain) and AD (activating domain) fusions on dropout plates.
(C) Quantification of β-gal activity in yeast cells containing various combinations of plasmids. Data shown are the average from triplicate assays.
(D) The expression of LptA and LptC proteins in yeast cells. Yeast cells harboring the LptA and LptC fusion plasmids were used to prepare protein samples and the expression was detected using anti-HA and anti-Myc antibodies.

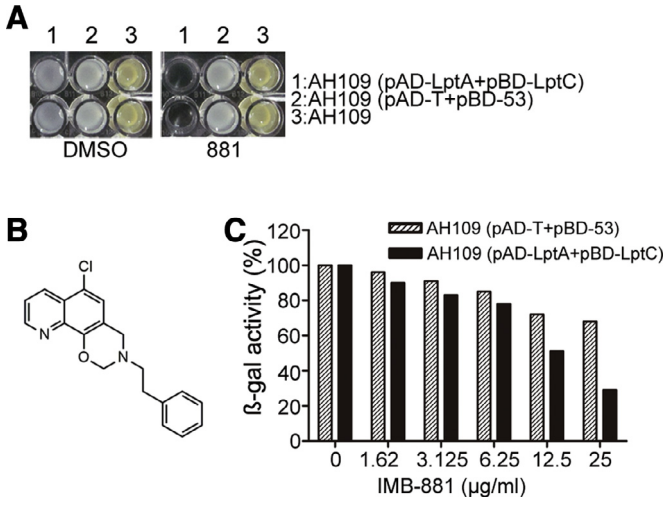

Fig. 3. Identification of compounds that block the LptA–LptC interaction.
(A) The growth inhibition of yeast cells by compound IMB-881. AH109 yeast strains with indicated plasmids were inoculated into SD –Leu/–Trp/–Ade/–His dropout medium in 96-well plates, while AH109 strain without any plasmid was inoculated into YPD medium. Compound IMB-881 was added into two wells for each strain at the concentration of 10 μg/mL, and their growth was examined after 24 hours of incubation at 30°C.
(B) The structure of compound IMB-881.
(C) The inhibition of β-gal activity of AH109 cells (pAD-LptA + pBD-LptC) by compound IMB-881 at various concentrations. Strain AH109 (pAD-T + pBD-53) was used as a control. The values represent the percentage of β-gal activity in cells treated with IMB-881 over that in untreated cells. β-gal activity was examined in the presence of compound IMB-881 at the concentrations of 1.62, 3.125, 6.25, 12.5, and 25 μg/mL.

CM5 to NTA. It is reasoned that His-tagged LptA and LptC exhibit stronger affinity to NTA sensor chips. However, the NTA sensor chip could not be used to assess LptA-LptC interaction as both are His-tagged proteins. Thus, a CM5 sensor chip was coated with LptA to determine whether compound IMB-881 abolishes LptA-LptC interaction. The study first exposed IMB-881 to LptA-coated sensor chip, injected purified LptC protein, and then examined the change of response units. The sensorgrams showed that IMB-881 significantly reduced the response to LptC injection (Fig. 4.D). These data collectively demonstrate that compound IMB-881 disrupts LptA-LptC interaction by binding to LptA.

3.4. Escherichia coli *cells treated with compound IMB-881 show filament morphology and abnormal LPS distribution*

Escherichia coli mutant cells lacking Lpt proteins exhibit striking alterations in envelope morphology, such as abnormal membrane structures and accumulation of 'extra' membrane material in the periplasm [11]. In addition, *E. coli* cells lacking some of the Lpt proteins show filament growth, presumably due to cell division failure [32]. If compound IMB-881 disrupts the interaction between LptA and LptC, *E. coli* cells treated with IMB-881 will be defective in LPS transport and show similar phenotypes as mutants lacking Lpt proteins. To test this possibility, *E. coli* cells were incubated in the presence of IMB-881 to examine the cell morphology with light microscopy as well as the membrane structures with transmission electron microscopy (TEM). As shown in Fig. 5. A, the cells treated with 6.25 μg/mL IMB-881 became elongated and showed filament growth over time. Moreover, in cells incubated with IMB-881, extra membrane materials were accumulated, and multilayered membranous bodies were observed in the periplasm using

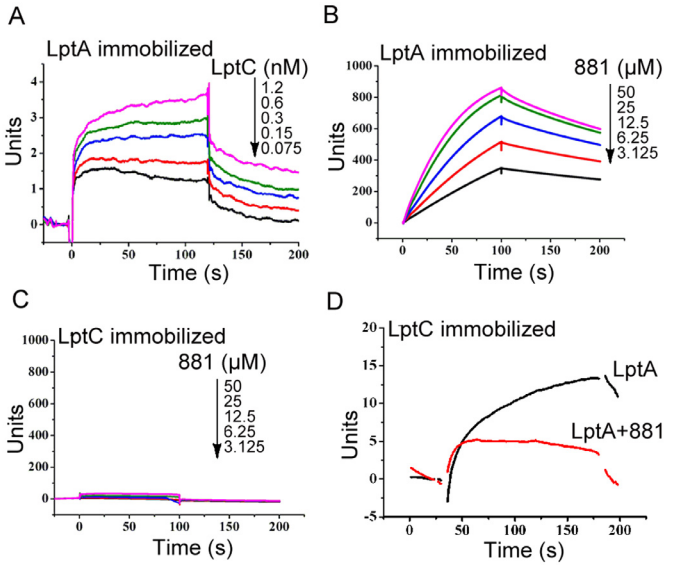

Fig. 4. Surface plasmon resonance analysis for the binding of compound IMB-881 with LptA and LptC proteins and the effect of IMB-881 on LptA–LptC interaction.
(A) Demonstration of LptA–LptC interaction by SPR. LptA was immobilized on the CM5 sensor chip, while LptC at series concentrations, ranging 0.075–1.2nM, was passed through the chip. The change in response units is shown.
(B and C) The binding of compound IMB-881 to LptA or LptC protein. Solutions with series concentrations of IMB-881 (3.13–50 μM) were injected into the chamber with NTA sensor chip coated with LptA (B) or LptC (C). The change in response units is shown.
(D) Compound IMB-881 blocked LptA–LptC interaction by binding to LptA protein. LptA proteins (10 μg/mL) preincubated with or without compound IMB-881 (12.5 μM) was injected into a chamber with a LptC-coated CM5 sensor chip. The change of response units was measured over time.

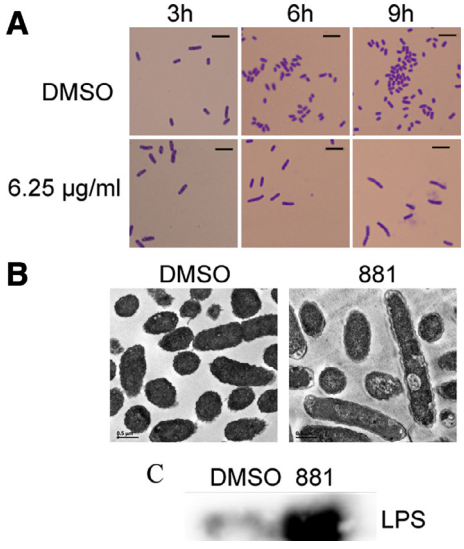

Fig. 5. *Escherichia coli* cells treated with compound IMB-881 show morphological change and accumulate LPS in the periplasmic space. *Escherichia coli* cells were treated with compound IMB-881 at concentrations of 6.25 μg/mL or DMSO at 37°C and then collected every hour for morphological examination.
(A) The morphology changes of *Escherichia coli* cells treated with IMB-881 after examination with a light microscope. Scale bars, 5 μm.
(B) The morphological alternation of *Escherichia coli* cells treated with IMB-881 for 9 h at 37°C after examination with transmission electron microscopy (TEM). Membrane materials were accumulated and multilayered membranous bodies were found in the periplasmic space in cells treated with compound IMB-881. Scale bars, 0.5 μm.
(C) The distribution of LPS in the periplasm. *Escherichia coli* cells were treated as described above and then the membrane fraction in periplasmic space was separated for the examination of LPS levels using western blotting with anti-LPS antibody.

TEM (Fig. 5.B). To determine the level of LPS in periplasm, the periplasm fractions of *E. coli* cells were obtained using a previously described method [28]. The level of LPS was detected by western blotting using the anti-LPS monoclonal antibody. Strikingly, the LPS level in the periplasm space from *E. coli* cells treated with IMB-881 (6.25 μg/mL) significantly increased compared to control cells (Fig. 5.C). The abnormal cell morphology and increased accumulation of LPS in the periplasm in drug-treated *E. coli* suggest that compound IMB-881 inhibits LPS transport.

3.5. LptA-LptC interaction is likely the target of compound IMB-881 in vivo

Because LptA–LptC interaction is essential for LPS transport, it was speculated that disruption of this interaction would inhibit the growth of *E. coli* cells. Indeed, a previous study showed that IMB-881 inhibits the growth of *E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa*), although the mechanism remains unclear [33]. Since the current study used *E. coli* ATCC 25922 to clone the genes encoding LptA and LptC proteins for this drug screen, we first determined the MIC of compound IMB-881 against this species, and IMB-881 showed an MIC of 6.25 μg/mL for ATCC 25922, which is similar to the previous report. Consistently, the MIC against *P. aeruginosa* was > 32 μg/mL. The MIC range for the clinical carbapenem-resistant *E. coli* strains was 6.25–12.5 μg/mL. For the clinical multidrug-resistant (MDR) *E. coli* strains, the MICs ranged 12.5–25 μg/mL (Table 1). Because of Lpt system conservation in Gram-negative bacteria, the growth inhibition of other strains by compound IMB-881 was also detected. Carbapenem is an antibiotic that kills bacteria by inhibiting cell wall synthesis and is widely used for treating infections caused by multidrug-resistant bacteria. The MICs of IMB-881 against clinical carbapenem-resistant *Enterobacter Cloacae* strains ranged 12.5–25 μg/mL. For some clinical *Acinetobacter baumannii* MDR strains, the MICs ranged 12.5–50 μg/mL. For a clinical carbapenem-resistant *Klebsiella pneumoniae* strain, the MIC was 12.5 μg/mL, but for some clinical MDR *Klebsiella pneumoniae* strains, the MICs were 25 μg/mL or 50 μg/mL (Table 2).

Next, this study assessed the mode of action of compound IMB-881 against Gram-negative bacteria. We examined the viability of *E. coli* over time in the presence of various concentrations of compound IMB-881. In the presence of 1 × MIC of IMB-881, the number of colonies remained constant during the time course for 5 hours and moderately increased afterwards, but the number was obviously less than that treated with DMSO. In contrast, the presence of compound IMB-881 at concentrations of 2 × or 4 × MIC, no colonies were observed after treatment for 8 and 6 hours, respectively, suggesting a strong bactericidal activity of IMB-881 (Fig. 6). Therefore, compound IMB-881 exhibits bacteriostatic and bactericidal activities at different concentrations.

If IMB-881 inhibits the growth of *E. coli* by binding to LptA protein and disrupting LPS transport, high level of LptA protein expression will alleviate the antibacterial activity of IMB-881. Therefore, the plasmids containing genes encoding LptA or LptC were constructed and introduced into *E. coli* BL21. Their expression was confirmed by western blotting (Fig. S1). For IMB-881, the MIC against the *E. coli* with a control vector was 6.25 μg/mL, but the MIC for BL21 overexpressing LptA was 12.5 μg/mL. Although the difference was not dramatic, this experiment was repeated six times and the results were consistent. In contrast, the MIC for the strains expressing LptC was the same as the control: 6.25 μg/mL. To clarify if the increase in MIC is specific to IMB-881, the effect of LptA and LptC overexpression on the MIC of other antibiotics, ciprofloxacin and ceftriaxone sodium, was also examined. For these antibiotics, the MICs against the *E. coli* with a control vector were 0.08 μg/mL and 0.16 μg/mL, respectively. However, the MICs

Table 1
The MICs (μg/mL) of IMB-881 against *Escherichia coli, Enterobacter cloacae* and *Klebsiella Bojun* strains.

Bacteria	A1929	A2291	A2370	A2388	A2392	A2451	25922	A2439	A2260	A2271
IMB-881	12.5	12.5	25	12.5	12.5	6.25	6.25	12.5	25	12.5

Strains A1929, A2291, A2370, A2388, A2392, and A2451 were all clinical carbapenem-resistant *Escherichia coli* stains. Strain 25922 is *Escherichia coli* ATCC25922 from ATCC. Strains A2439 and A2260 were clinical carbapenem-resistant *Enterobacter cloacae* strains. Strain A227 is a clinical isolate of carbapenem-resistant *Klebsiella pneumoniae*.

Table 2
The MICs (μg/mL) of IMB-881 against MDR *Klebsiella pneumoniae* and *Acinetobacter baumannii* strains.

Bacteria	Ab1	Ab2	Ab3	Ab4	Ab5	Ab6	Kp1	Kp2	Kp3	Kp4
IMB-881	12.5	12.5	25	12.5	50	12.5	50	50	25	50

Strains Ab1, Ab2, Ab3, Ab4, Ab5, and Ab6 were all clinical multidrug resistance (MDR) *Acinetobacter baumannii* isolates. Strains Kp1, Kp2, Kp3, and Kp4 were all clinical MDR *Klebsiella pneumoniae* isolates.

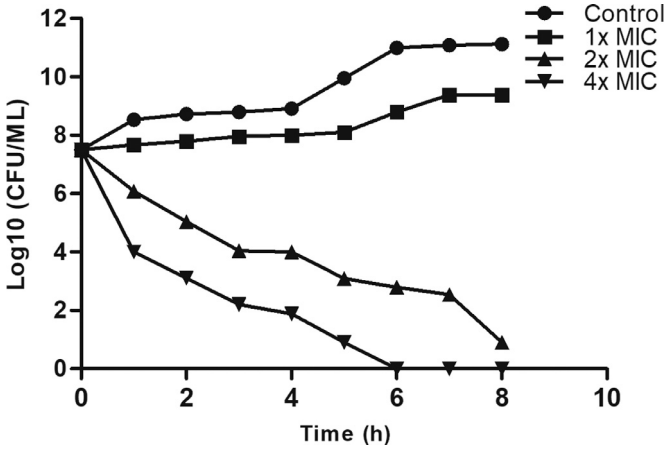

Fig. 6. Compound IMB-881 exhibited bacteriostatic and bactericidal activities. An initial inoculum of around 1×10^7 CFU/mL of *Escherichia coli* ATCC 25922 was grown in LB medium containing various concentrations of compound IMB-881. The MIC of IMB-881 for this strain is 6.25 μg/mL. The colony numbers were counted after incubation in the presence of IMB-881 at 1-, 2- and 4-fold of MICs.

of these two antibiotics against BL21 overexpressing LptA were 0.02 μg/mL and 0.04 μg/mL, respectively, showing a clear decrease compared to the control. Collectively, these data indicate that LptA is likely the target of compound IMB-881 in vivo, and the disruption of LptA-LptC interaction may contribute to the bacteriostatic activity of this compound.

4. Discussion

Lipopolysaccharide (LPS) provides the integrity of OM of Gram-negative bacteria and acts as a permeability barrier for hydrophobic antibiotics. Because LPS is unique to Gram-negative bacteria, great effort has been made to isolate antibiotics targeting LPS synthesis [34,35]. For example, the discovery of small-molecule inhibitors for LpxC, the enzyme responsible for the first committed step in the biosynthesis of lipid A, represents a strategy to identify new antibiotics that specifically kill Gram-negative bacteria [34]. Because LPS is synthesized in cytoplasm, its transport is essential for LPS assembly at the OM out-leaflet. However, only a family of peptidomimetic antibiotics was identified that targets LptD and blocks LptD-dependent LPS transport and assembly in OM [35]. The current study first used the yeast two-hybrid system to confirm the interaction of *E. coli* proteins LptA and LptC. With this system, it identified compound IMB-881, which blocks LptA-LptC interaction. The SPR results suggest that this compound binds to LptA to block this interaction.

This study found that bacteria overexpressing LptA show two-fold increase of MIC for IMB-881. It is understood that the two-fold difference in MIC between the *E. coli* strains with a control vector and LptA overexpression plasmid is not significant, but this result was reproducible, as it was repeated six times. On the other hand, the bacteria strains overexpressing LptA protein exhibited more sensitivity to ciprofloxacin and ceftriaxone sodium, thus the MIC increase was specific to IMB-881. It is likely that high levels of LptA proteins decrease the efficiency of IMB-881 inside bacteria, because of the interaction between LptA and IMB-881. On the other hand, overexpression of LptA might also be harmful to the growth of these strains, which could increase the sensitivity to other antibiotics such as ciprofloxacin and ceftriaxone sodium.

The current study further showed that compound IMB-881 exhibits bacteriostatic activity against Gram-negative bacteria at low concentration ($1 \times$ MIC) and bactericidal activity at high concentrations. It is reasoned, based on the following observations, that compound IMB-881 inhibits bacteria growth by disrupting LPS transport. First, the results indicate that this compound blocks the interaction between LptA and LptC, which is essential for LPS transport. The SPR technique was used to demonstrate that IMB-881 disrupts LptA-LptC interaction through its specific binding to LptA. Strikingly, the alternation of *E. coli* envelope treated with IMB-881 at low concentration (6.25 μg/mL) is similar to mutants lacking LptA or other Lpt proteins, as evidenced by the filament morphology, abnormal membrane structure, and accumulation of extra membrane material in the periplasm [29,32]. Moreover, overexpression of LptA in *E. coli* increases the MIC of IMB-881 twofold. Together, these results suggest that the in vivo target of IMB-881 is likely LptA, and its association with LptA disrupts LPS transport across the periplasm and subsequently blocks LPS assembly at the OM. Therefore, compound IMB-881 represents a unique class of antibacterial agents that may impair LPS transport. It was also noticed that IMB-881 kills *E. coli* cells within 6 hours at $4 \times$ MIC, which is different from other compounds targeting LPS synthesis such as LpxC inhibitors [34]. One possibility is that IMB-881 has other targets in addition to LptA-LptC interaction, but the possibility that the complete block of LptA-LptC interaction by IMB-881 at high concentrations contributes to bactericidal activity cannot be excluded.

Since compound IMB-881 blocks LptA-LptC interaction, it is speculated that the disruption of this interaction prevents LPS transport. Previous work has shown that *E. coli* LptA monomers are packed as a linear filament, leading to the hypothesis that oligomers of LptA may be required to bridge the IM and OM to facilitate LPS export [15]. Therefore, another untested possibility is

that the interaction of compound IMB-881 with LptA prevents its oligomerization and blocks LPS transport in the periplasm. It is also possible that this compound blocks LptA-LPS interaction or affects the binding of LptA with other Lpt proteins. Further experiments are needed to test these possibilities, but the current data support the conclusion that LptA is likely one of the in vivo targets of IMB-881. In the future, the same strategy will be used to isolate more compounds that target LptA-LptC interaction in order to obtain antibiotics that impair LPS transport in Gram-negative bacteria.

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Declarations

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Competing Interests

None.

Ethical Approval

Ethical Approval was not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.11.016.

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