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Original Article

# Potentials of organic tellurium-containing compound AS101 to overcome carbapenemase-producing *Escherichia coli*

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**KEYWORDS**
Carbapenem resistance;
AS101;
*Escherichia coli*;
Mouse sepsis model

**Abstract** *Background:* The issue of carbapenem-resistant *Escherichia coli* was aggravated yearly. The previous studies reported the varied but critical epidemiology of carbapenem-resistant *E. coli* among which the carbapenemase-producing strains were regarded as one of the most notorious issues. AS101, an organic tellurium-containing compound undergoing clinical trials, was revealed with antibacterial activities. However, little is known about the antibacterial effect of AS101 against carbapenemase-producing *E. coli* (CPEC).
*Materials and methods:* The minimum inhibitory concentration (MIC) of AS101 against the 15 isolates was examined using a broth microdilution method. The scanning electron microscopy, pharmaceutical manipulations, reactive oxygen species level, and DNA fragmentation assay were carried out to investigate the antibacterial mechanism. The sepsis mouse model was employed to assess the *in vivo* treatment effect.
*Results:* The $bla_{NDM}$ (33.3%) was revealed as the dominant carbapenemase gene among the 15 CPEC isolates, followed by the $bla_{KPC}$ gene (26.7%). The MICs of AS101 against the 15 isolates ranged from 0.5 to 32 μg/ml, and 99.9% of bacterial eradication was observed at 8 h, 4 h, and 2 h for 1×, 2×, and 4 × MIC, respectively. The mechanistic investigations suggest that AS101 would enter the bacterial cell, and induce ROS generation, leading to DNA fragmentation. The *in vivo* study exhibited that AS101 possessed a steady treatment effect in a sepsis mouse model, with an up to 83.3% of survival rate.
*Conclusion:* The *in vitro* activities, mechanisms, and *in vivo* study of AS101 against CPEC were unveiled. Our finding provided further evidence for the antibiotic development of AS101.
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## Introduction

*Escherichia coli* as a member of *Enterobacterales*, is one of the majorly causative agents for infections, often with multidrug resistance.[1,2] Although *E. coli* was regarded as a commensal bacteria in the human gut, some strains could become extraintestinal pathogenic *E. coli* (ExPEC) once getting the virulence factors.[3] Owing to the increased antibiotic resistance in *E. coli*, carbapenems were considered as the treatment option to treat multidrug-resistant *E. coli* infections. However, the emergence of carbapenem resistance in *E. coli* has been reported worldwide in the past decade.[4] The world health organization (WHO) has also announced the carbapenem-resistant *Enterobacterales* as the critical priority for antibiotic development, highlighting the issue of carbapenem-resistant *E. coli*.[5] The previous report, in which 5796 clinical isolates of *E. coli* were collected from 2002 to 2017, revealed a 1% carbapenem-resistant rate,[6] whereas another study from India recently described that 81 of 279 *E. coli* (29%) isolated from calves were resistant to at least one carbapenem.[7] The studies implied the varied but critical epidemiology of carbapenem-resistant *E. coli* among which the carbapenemase-producing strains were regarded as one of the most notorious issues due to the highly-transmittable carbapenemase-harboring plasmid.[8] In this scenario, a new antibiotic for carbapenem-resistant crises is urgently needed.

Ammonium trichloro (dioxoethylene-O,O′) tellurate (AS101, MW≒312 Da.), as an immunomodulatory agent, could enhance the secretions of cytokines (IL-1α, IL-2, TNFα, and colony-stimulating factor).[9,10] The previous studies have reported the anti-virus and anti-parasite effects for AS101.[11,12] The low toxicity of AS101 was revealed with a 50% cytotoxic concentration ($CC_{50}$) in Vero cell of 145 μg/ml and a 50% lethal dose ($LD_{50}$) in mice of 10 mg/kg.[11,13] The ClinicalTrials.gov website recorded several clinical trials of AS101 in different applications, such as phase I for HIV infections (NCT00001006, completed in 2012), phase I/II studies for external genital warts (NCT01555112, completed in 2013), and phase I/II studies for aging macular degeneration (NCT03216538, currently ongoing). Encouraged by these efforts, we attempted to re-evaluate AS101 as an antimicrobial agent. To address the issue of carbapenem resistance, our recent studies reported that AS101 was noticed to possess the potential to treat the carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and *Acinetobacter baumannii* (CRAB).[14,15] The MIC ranges of AS101 against the carbapenem-resistant *K. pneumoniae* and *A. baumannii* clinical isolates were <0.5—32 μg/ml and 0.5—32 μg/ml, respectively. Furthermore, AS101 demonstrated robust treatment effects in the CRKP and CRAB infectious sepsis mouse models, with better survival rates compared to the clinical standard treatments. According to previous pharmaceutical research, the antibacterial activity of an antibiotic against one bacterium could not be speculated depending on the observation of that against another bacterium.[16] Different from other bacterial species, the variable virulence factors in *E. coli*, such as ExPEC, could lead to more severe infections, especially the ST131 with carbapenem resistance urgently threatening global health.[17] To this end, even though some evidence supported the antibacterial activity of AS101, little is known about its antibacterial effect on carbapenemase-producing *E. coli*. In this study, we sought to investigate the *in vitro*, *in vivo*, and mechanistic characteristics of AS101 against carbapenemase-producing ST131 *E. coli* (CPEC) clinical isolates.

## Materials and methods

### Bacterial collection

A total of 15 CPEC clinical isolates were collected between 2012 and 2015 as part of a nationwide (Taiwan) surveillance study.[18] All isolates were detected with a carbapenemase gene via PCR and were resistant to at least one of the carbapenems.

### Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of the 15 isolates was performed using the broth microdilution method (Sensititre, Trek Diagnostic Systems, Cleveland, OH, USA), including ampicillin, aztreonam, ceftazidime, cefazolin, ciprofloxacin, ertapenem, cefepime, cefoxitin, cefotaxime, ceftriaxone, gentamicin, imipenem, levofloxacin, meropenem, trimethoprim/sulfamethoxazole, and piperacillin-tazobactam. The interpretations of the susceptibility results were according to CLSI guidelines.[19] The broth microdilution of colistin against the 15 isolates was carried out manually, and the resistance was defined as MIC $\geq$4 μg/ml following the criteria recommended by CLSI in 2023.

### Bacterial genotyping

The *Xba*I (New England BioLabs, Ipswich, MA)-digested DNA fragments were subjected to the pulsed-field gel electrophoresis (PFGE) using a CHEF Mapping apparatus (Bio-Rad Laboratories, Hercules, CA) as described in the previous study.[20] The dendrogram of the pulsotype relationship was constructed in GelComparII software using methods of Dice similarity and unweighted pair group method with arithmetic mean (UPGMA). The isolates that exhibited 80% dendrogram similarity were assigned to the same cluster.[20] Primer sets used for the multiple locus sequencing typing (MLST) were available at enterobase.warwick.ac.uk/species/ecoli/allele_st_search. The sequencing results of seven housekeeping genes: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* were employed to determine the sequence type for the 15 CPEC isolates.

### Detection of β-lactamase and virulence genes

The polymerase chain reaction (PCR) detection was performed to detect the presence of ESBL genes ($bla_{SHV}$, $bla_{TEM}$, $bla_{OXA}$, $bla_{CTX\text{-}M\text{-}G1}$, $bla_{CTX\text{-}M\text{-}G2}$, and $bla_{CTX\text{-}M\text{-}G9}$), plasmid-mediated AmpC genes ($bla_{DHA}$ and $bla_{CMY}$) and carbapenemase genes ($bla_{KPC}$, $bla_{NDM}$, $bla_{IMP}$, $bla_{NMC}$, $bla_{SME}$, $bla_{VIM}$, $bla_{SPM\text{-}1}$, $bla_{GIM\text{-}1}$, $bla_{SIM\text{-}1}$, $bla_{IMI}$, $bla_{GES}$, and $bla_{OXA\text{-}48}$).[21,22] The virulence genes of extraintestinal pathogenic *E. coli* were also detected, including *papC*, *afaBC*, *sfa*, *iutA*, *kpsMT* II.[23] According to the previous study,[24] the isolate with any two of the virulence genes we detected was defined as extraintestinal pathogenic *E. coli*. All analyses were performed with corresponding positive controls.

### Minimum inhibitory concentration (MIC) of AS101

The minimum inhibitory concentrations of AS101 against 15 CPEC clinical isolates were determined using a broth microdilution method as previously described.[14] In brief, AS101 (Development Center for Biotechnology, Taiwan) was dissolved in 99% ethanol and serially 2-fold diluted in brain-heart infusion (BHI) broth to concentrations ranging from 0.5 to 32 μg/ml, with a final ethanol concentration of 5% in wells. The starting bacterial inoculum was $5 \times 10^5$ colony-forming units per ml (CFU/ml) in wells. The absorbance was detected before and after 16–18 h incubation at 37 °C using a microplate reader, and bacterial growth was determined via the change of the absorbances. For the assays of pharmacological manipulations, agents (4 mM of EDTA,[25] 320 mM of mannitol,[26] 10 mM of calcium chloride, or 10 mM magnesium chloride[27]) were added while examining a broth microdilution.[14]

### Time-kill assays

The antibacterial activity of AS101 against KPC-2-producing *E. coli* CRE-415 was revealed using time-kill assays as described in a previous study.[28] $5 \times 10^5$ CFU/ml of log-phase *E. coli* CRE-415 was incubated with 1×, 2× or 4 × MIC of AS101 at 37 °C, and the 5% of ethanol served as a control. Samples were harvested at 0, 2, 4, 8, and 24 h post-incubation, were serially 10-fold diluted in 1 × PBS, and were plated onto LB agar. The viable numbers of samples were counted after 18 h incubation at 37 °C and used to construct the time-kill curves. Amikacin and tigecycline were used as bactericidal and bacteriostatic agent controls, respectively. A reduction $>3\ \log_{10}$ (>99.9%) of total CFU/ml compared to the initial inoculum was regarded as a bactericidal activity; if not, a bacteriostatic activity.

### Scanning electron microscopy (SEM)

Scanning electron microscopy procedures were performed as previously described.[29] The bacterial cells of *E. coli* CRE-415 were treated with 4 μg/ml of AS101 (the 1 × MIC) for an hour. After sample collection, fixation, and dehydration, 5 μl of the resulting sample were dripped onto a sample platform and let dry for 2 weeks. The sample platform was coated before observation, and the micrographs were captured using the scanning electron microscope (JEOL, JSM-5300).

### Reactive oxygen species (ROS) level

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidative stress detection method was employed to detect the cellular ROS level in *E. coli* CRE-415.[30] Bacterial cells were incubated with 100 μM DCFH-DA (Sigma–Aldrich) in BHI broth for 2 h. The DCFH-DA-treated cells were washed with 1 × PBS, resuspended in BHI broth, and adjusted to appropriate densities. Following, cells were treated with 1×, 2× or 4 × MIC of AS101 at 37 °C for 1 h, and those treated with 5% ethanol were used as a control. The fluorescent intensity was detected using a spectrofluorometric

reader (Plate Chameleon II model 425-155, Hidex) at 500 and 530 nm wavelengths for excitation and emission, respectively. After detection, samples were subjected to serial 10-fold dilutions for the viable bacterial counts, which were applied to the normalization of results. All experiments were performed in triplicate.

## DNA fragmentation assay

The DNA fragmentation assay was carried out as previously described.[31] Briefly, $10^9$ CFU/ml of the bacterial cell was treated with 16, 32, or 64 μg/ml of AS101 in 96-well microtiter plates at 37 °C for 1 h. Samples were collected and analyzed using PFGE as described above. The bands of DNA fragments were further quantified using Image J software.

## Animal study

The animal proposals were approved by the Kaohsiung Medical University (KMU) Institutional Animal Care and Use Committee (No. 108124), and all procedures were executed under the KMU institutional guidelines. Specific pathogen-free (SPF) 6- to 8-week-old male ICR (CD1) mice were purchased from Lasco Biotechnology (Taiwan) and housed in SPF units in the KMU Laboratory Animal Center for at least 1 week prior to experiments. Animals were randomly grouped, and to maximize blinding, all animal infection, treatment, and tissue processing procedures were performed by two independent researchers. All animal work was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility.

A sepsis mouse model was established according to previous studies.[32,33] Animals were inoculated with a lethal dose (ca. $10^7$ CFU) of *E. coli* CRE-415 via intraperitoneal (i.p.) injection. The infected mice were treated with 20 mg/kg/day of colistin (QID) (colistin methanesulfonate, CMS) (Santa Cruz), 1.67 or 3.33 mg/kg of AS101 (QID), or a PBS vehicle 30 min post-infection.[11,14,15,34] Mouse survival was recorded every 6 h over 3 days.

The organ samples were collected using the same sepsis mouse model to investigate the bacterial load on the liver, spleen, and kidney. Mice were euthanized 18 h post-infection, and the organs were harvested, placed in 2 ml of sterile PBS, and held on ice before homogenization. The homogenized organ samples were serially diluted and plated onto LB agar with 4 μg/ml of meropenem. The colonies were enumerated and normalized with the organ weight. All experiments were repeated at least 3 times.

## Statistical analysis

The profiles of the antimicrobial susceptibility and ESBL genes for 15 isolates tested in this study were visualized using the "heatmap.plus" package in RStudio software version 1.1.453. The DNA fragmentation assay was quantified using Image J software. The quantitative data of the time-kill curve, ROS level, and DNA fragmentation assays were visualized using Prism 7 (GraphPad, USA). The Kaplan—Meier curve of mouse survival was constructed also using Prism 7 software and analyzed using Mantel—Cox log-rank tests. The continuous variables were compared using Student's *t*-test.

# Results

## Characterization of 15 CPEC clinical isolates

The genetic typing results demonstrated a close phylogenetic relationship between the isolates CRE-792 and CRE-845, with the same pulsotype and sequence type (Fig. S1). Among the 15 isolates, three isolates were classified as ST131 (3/15, 20%), two were ST10 (2/15, 13.3%) and seven different ST was single isolate, respectively. Among 17 antibiotics we tested for the 15 isolates, 15 of them demonstrated low susceptibilities (≤20%), including ampicillin (0% susceptible), aztreonam (6.7%), ceftazidime (0%), cefazolin (0%), cefepime (6.7%), cefoxitin (0%), ceftriaxone (0%), cefotaxime (0%), imipenem (6.7%), ertapenem (0%), meropenem (13.3%), piperacillin-tazobactam (0%), ciprofloxacin (13.3%), levofloxacin (20%), and trimethoprim/sulfamethoxazole (20%) (Fig. S2). A moderate susceptibility was found for gentamicin against the 15 isolates, with a susceptible rate of 40%, whereas colistin represented 100% susceptibility. Among the carbapenemase genes in 15 isolates, $bla_{NDM}$ (5/15, 33.3%) was the dominant, followed by $bla_{KPC-2}$ (4/15, 26.7%), $bla_{VIM}$ (3/15, 20.0%), $bla_{OXA-48}$ (2/15, 13.3%), and $bla_{IMP}$ (1/15, 6.7%) (Table 1). Of other β-lactamase genes we detected, $bla_{CTX-M}$ (11/15, 73.3%) was dominant, followed by $bla_{OXA-1}$ (8/15, 53.3%), $bla_{TEM}$ (6/15, 40.0%), $bla_{CMY}$ (6/15, 40.0%), $bla_{SHV}$ (2/15, 13.3%), and $bla_{DHA}$ (1/15, 6.7%) (Fig. S3). The detection of virulence genes showed that two of 15 isolates (2/15, 13.3%) were detected with *papC* and *iutA* genes (Table 1), suggesting to be extraintestinal pathogenic *E. coli* isolates.

## Antibacterial activities of AS101

The MICs of AS101 against the 15 isolates ranged from 0.5 to 32 μg/ml, with the $MIC_{50}$ and $MIC_{90}$ of 4 μg/ml and 32 μg/ml, respectively. According to the previous epidemiological study in Taiwan,[18] the KPC-2 was the most prevalent carbapenemase in Taiwan. The KPC-2-producing ST131 CRE-415 carrying *iutA* virulence factor, was selected for further analyses (Table S1). The time-kill curves of amikacin and tigecycline against CRE-415 reasonably represented the bactericidal and bacteriostatic activities, respectively (Fig. 1A and B). As shown in Fig. 1C, AS101 demonstrated a remarkable bactericidal activity against *E. coli* CRE-415, with bacterial reduction of 98.54%, 99.59%, and 99.93% for 1×, 2×, and 4 × MIC, respectively, within 2 h. The 99.9% decreases in the bacterial count were found at 8 h, 4 h, and 2 h for 1×, 2×, and 4 × MIC, respectively, suggesting AS101 as a bactericidal agent. According to the short-term time-kill curve of AS101 (Fig. S4), the significant bacterial elimination was first observed 1 h after treatment at 1 × MIC. Thus, the condition was selected for further SEM observation. The micrographs of CRE-415 showed a smooth and intact surface of the bacterial cells (Fig. 2A and B). After being treated with 1 × MIC (4 μg/ml) of AS101, the bacterial cells were found to elongate and with pores on the surface,

**Table 1** Characterization and AS101 MICs of 15 carbapenemase-producing *E. coli* isolates in this study.

| Strain | AS101 MIC (μg/ml) | Carbapenemase | Virulence factors | | | | |
|---|---|---|---|---|---|---|---|
| | | | papC | sfa | afaBC | iutA | *kpsMT* II |
| CRE-354 | 0.5 | KPC-2 | - | - | - | - | - |
| CRE-381 | 32 | NDM | - | - | - | - | - |
| CRE-415 | 4 | KPC-2 | - | - | - | + | - |
| CRE-621 | 4 | NDM | - | - | - | - | - |
| CRE-649 | 8 | NDM | - | - | - | + | - |
| CRE-792 | 4 | KPC-2 | - | - | - | - | - |
| CRE-845 | 2 | KPC-2 | - | - | - | - | - |
| CRE-908 | 0.5 | NDM | - | - | - | - | - |
| CRE-988 | 32 | NDM | - | - | - | - | - |
| CRE-1176 | 32 | VIM | - | - | - | - | - |
| CRE-1202 | 8 | OXA-48 | - | - | - | - | - |
| CRE-1261 | 4 | VIM | - | - | - | + | - |
| CRE-1534 | 2 | IMP | + | - | - | + | - |
| CRE-1764 | 16 | VIM | - | - | - | - | - |
| CRE-1811 | 32 | OXA-48 | + | - | - | + | - |

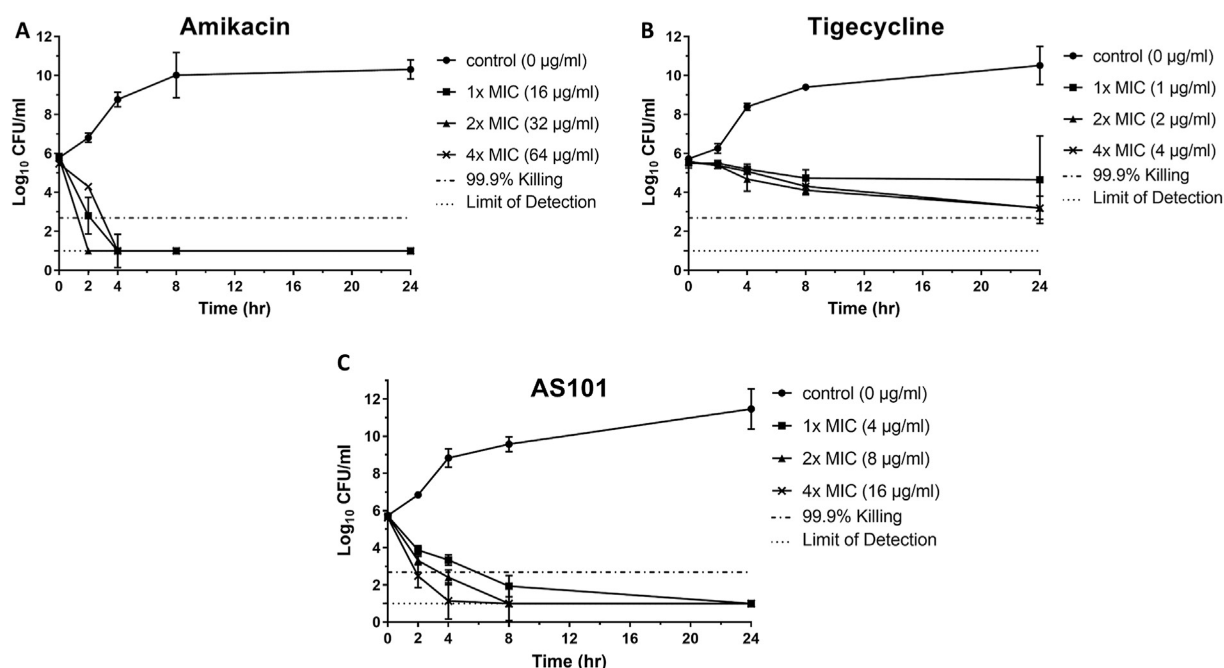


**Figure 1.** **Time-kill kinetic assays of AS101 against carbapenemase-producing *E. coli* clinical isolate CRE-415.** With 1 × MIC (filled squares), 2 × MIC (filled triangles), or 4 × MIC (cross) of **A**, amikacin; **B**, tigecycline; **C**, AS101; or untreated (control, filled circle), the survival of CPEC CRE-415 was measured at 2, 4, 8 and 24 h. The dotted-dashed lines represent the 99.9% eradication compared to the beginning inoculum, and the dotted lines indicated the limit of detection. CFU, colony-forming unit.

suggesting the antibacterial stress from AS101 against CRE-415 (Fig. 2C and D).

## Mechanistic studies

To investigate the antibacterial mechanism of AS101, the chemical manipulations were performed using 4 mM of EDTA to increase membrane permeability, 320 mM of mannitol as a ROS scavenger, or 10 mM of calcium chloride/magnesium chloride to change the outer membrane charge (Table 2). Compared to the untreated group (MIC = 4 μg/ml), the 4 mM of EDTA decreased the MIC to 0.125 μg/ml (32 folds), whereas the 320 mM of mannitol raised the MIC to 64 μg/ml (16 folds), implying that AS101 might enter the bacterial cell and lead to the accumulation of ROS. The cellular ROS levels were determined for the bacterial cells treated with 1× (4 μg/ml), 2× (8 μg/ml), 4 × MIC (16 μg/ml) of AS101, or the control treatment. As shown in Fig. 3, significant increases in ROS levels were found for cells treated with AS101 than the control, with a dose-dependent trend, suggesting the accumulation of ROS. Furthermore, the DNA fragmentation

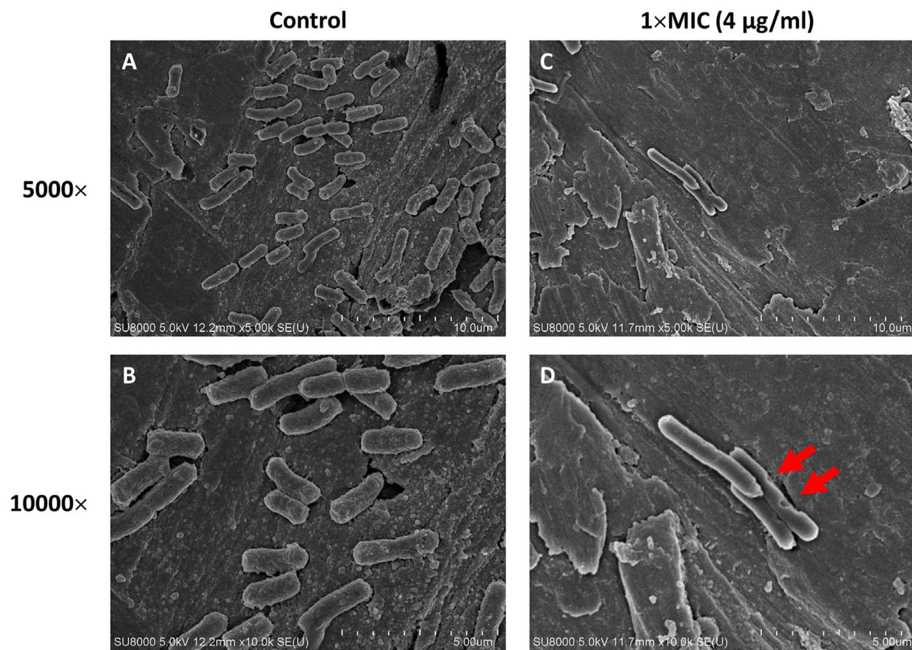


**Figure 2. Scanning electron microscopy (SEM) micrographs for AS101-treated CPEC CRE-415.** The control group showed the images of untreated bacteria captured at 5000× (**A**) and 10,000× (**B**) magnification; the bacteria treated with 1 × MIC (4 μg/ml) of AS101 were also recorded at 5000× (**C**) and 10,000× (**D**) magnification. The red arrows exhibited the leaking wrinkly surfaces for AS101-treated CPEC CRE-415.

**Table 2** AS101 MICs of KPC-2-producing *E. coli* CRE-415 with or without chemical manipulations.

| Chemical manipulation | Function | AS101 MIC (μg/ml) |
|---|---|---|
| Untreated | - | 4 |
| 4 mM EDTA | alteration of outer-membrane permeability | 0.125 |
| 10 mM $Ca^{2+}$ | alteration of outer-membrane charge | 4 |
| 10 mM $Mg^{2+}$ | alteration of outer-membrane charge | 2 |
| 320 mM Mannitol | ROS scavenger | 64 |

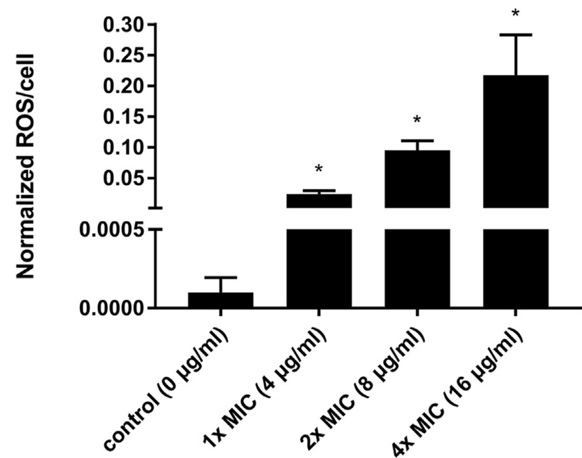


**Figure 3. ROS levels in CPEC CRE-415.** Following exposure to 1 × MIC (4 μg/ml), 2 × MIC (8 μg/ml), or 4 × MIC (16 μg/ml) of AS101, or control treatment (0 μg/ml), the intracellular ROS levels were measured using DCFH-DA assay. *, $p < 0.05$.

assays were carried out for the bacterial cells treated with 4× (16 μg/ml), 8× (32 μg/ml), 16 × MIC (64 μg/ml), 32 × MIC (128 μg/ml) of AS101, or the control treatment. The different degrees of DNA fragmentation were observed in the result of the pulsed-field gel electrophoresis (Fig. 4A). The amounts of DNA fragments were quantified and normalized by the control (Fig. 4B). The significantly increased amounts of DNA fragments were noticed for AS101 at all concentrations we tested, with a dose-dependent trend from 4× to 16 × MIC. The ROS and DNA fragmentation assays illustrated that AS101 could induce ROS accumulation and injure bacterial DNA.

## *In vivo* assessments

To evaluate the treatment effect of AS101, mice were exposed to a lethal dose of *E. coli* CRE-415 to establish a sepsis infection mouse model. All infected mice with the placebo treatment died 18 h post-infection (Fig. 5), whereas the 20 mg/kg/day of colistin methanesulfonate (CMS, colistin), which is susceptible against CRE-415 *in vitro* (Fig. S2) only rescued 33.3% (2/6) of the mice. Similar to the result of the CMS group, two of six mice (33.3%) receiving 1.67 mg/kg/day of AS101 survived. Remarkably, five of six mice (83.3%) were recovered by 3.33 mg/kg/day of AS101. The bacterial loads of the mice with placebo treatment were 7.60 ± 0.63, 7.11 ± 0.58, and 7.87 ± 0.31 $log_{10}$ CFU/ml on the liver, kidney, and spleen, respectively (Table 3). Compared to those of the

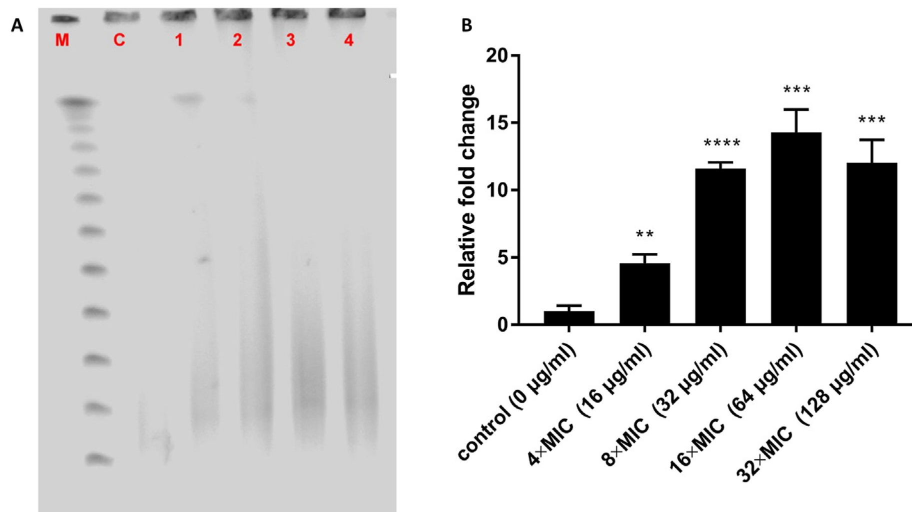


**Figure 4. DNA fragmentation assay for AS101-treated CPEC CRE-415.** (**A**) Bacteria treated with 4 × MIC (16 μg/ml) (lane 1), 8 × MIC (32 μg/ml) (lane 2), 16 × MIC (64 μg/ml) (lane 3), or 32 × MIC (128 μg/ml) (lane 4) of AS101, or control treatment (0 μg/ml) (lane C) were subjected to the pulsed-field gel electrophoresis. The Lambda PFG Ladder (N0341S, NEB) was utilized as the marker (lane M). (**B**) The triplicated results were further quantified using ImageJ software. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$.

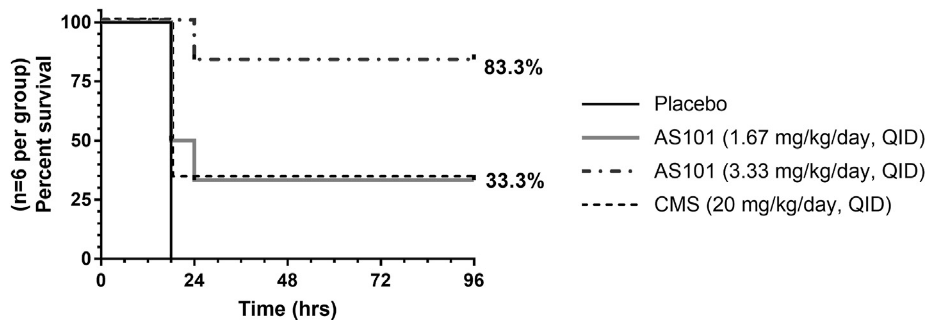


**Figure 5. ST131 CPEC CRE-415 lethally infected mice treated with AS101.** 6 of mice each group were intraperitoneally injected with $10^7$ CFU of CPEC CRE-415 and treated with placebo (PBS), CMS (colistin methanesulfonate), or AS101. CMS (colistin methanesulfonate) and AS101 treatments were quater in die.

**Table 3** Bacterial load in the liver, kidney, or spleen of the infected mice with different treatments.

| Group | Log CFU/g (mean ± SD) | | |
|---|---|---|---|
| | Liver | Kidney | Spleen |
| Placebo | 7.60 ± 0.63 | 7.11 ± 0.58 | 7.87 ± 0.31 |
| AS101 (1.67 mg/kg) | 4.2 ± 1.22 * | 3.95 ± 1.31 * | 4.13 ± 1.50 * |
| AS101 (3.33 mg/kg) | 3.01 ± 0.80 ** | 1.72 ± 1.59 ** | 3.70 ± 1.26 ** |
| CMS (20 mg/kg) | 5.87 ± 3.37 ns | 5.81 ± 2.70 ns | 7.14 ± 2.63 ns |

SD, standard deviation; *, $p < 0.05$; **, $p < 0.01$; ns, no significance.

placebo group, the bacterial loads on the liver, kidney, and spleen of the group treated with 1.67 mg/kg of AS101 significantly reduced to 4.2 ± 1.22 ($p < 0.05$), 3.95 ± 1.31 ($p < 0.05$), and 4.13 ± 1.50 ($p < 0.05$) $\log_{10}$ CFU/ml, respectively. The significant eradication of the bacterial loads was also observed dose-dependently for the group treated with 3.33 mg/kg of AS101, with those on the liver, kidney, and spleen of 3.01 ± 0.80 ($p < 0.01$), 1.72 ± 1.59 ($p < 0.01$), and 3.70 ± 1.26 ($p < 0.01$), respectively. Although no statistical significance was found for the bacterial loads of the group treated with CMS compared to that of the placebo group, the decreased trends were noticed for the bacterial loads on the liver and kidney. To sum up, AS101 could ameliorate the survival of the sepsis mice infected by *E. coli* by eradicating the bacterial titer on organs.

## Discussion

Due to the abuse of antibiotics, the antibiotic resistance in *E. coli* raised yearly, particularly carbapenems,[35] and thus, the World Health Organization (WHO) has announced the carbapenem-resistant *Enterobacterales* (previously *Enterobacteriaceae*) as the critical priority for the antibiotic development.[5] Among the carbapenem-resistant crises, the highly transmittable carbapenemase genes, including KPC, NDM, OXA-48, *etc*., have attracted many researchers' attention worldwide.[36–39] In a nationwide study in China, Zhang et al. analyzed 150 isolates of CPEC collected from 2014 to 2015.[40] Of these, 81 isolates were harbored by $bla_{NDM}$ (81/150, 49%), 65 by $bla_{KPC}$ (65/150, 40%), and 3 by $bla_{IMP}$ (3/150, 2%). Govindaswamy et al. collected 94 CPEC clinical isolates from a tertiary care hospital and investigated the prevalence of the carbapenemase genes.[41] Among the 94 isolates, $bla_{NDM\text{-}1}$ was the dominant and harbored in 58 isolates (61.7%), followed by $bla_{VIM}$ (29/94, 30.8%) and $bla_{KPC}$ (10/94, 10.6%). In a previous study reported in 2022, Iraq, 38 isolates of CPEC were collected between 2018 and 2019. Among those, 22 isolates carried $bla_{OXA\text{-}48}$ gene (22/28, 57.8%), 18 carried $bla_{PER}$ (18/28, 47.3%), and 6 isolates carried $bla_{KPC}$ (6/28, 15.7%). In the present work, among 15 CPEC clinical isolates, 5 were found with $bla_{NDM}$, and 4 isolates were detected with $bla_{KPC}$ (Table 1), agreeing with the report from China.[41] The tough issue underscored the urgent need for new antibiotics.

The current solution for the carbapenem-resistant *Enterobacterales* was β-lactam-β-lactamase inhibitor combinations, such as ceftazidime-avibactam, aztreonam-avibactam, imipenem-relebactam.[42–44] However, avibactam and relebactam shared similar chemical structures and inhibitory mechanisms against β-lactamases, and both can't inhibit the metallo-β-lactamases.[45] Furthermore, depending on the same antibacterial mechanism—β-lactam, some studies have reported the inhibitor-resistant β-lactamases and their increasing trend, leading the situation back to square one.[46,47] To this end, the development of a new class of antibiotics became a certain issue. Owing to the huge economic and time burdens of drug development, the concept of drug repurposing has been regarded as an attractive option.[48] Kwan et al. re-evaluated the anticancer agent, mitomycin C, as an antibacterial agent, with the MIC against *E. coli* ranging from 1 to 2 μg/ml.[49] In their work, mitomycin was found to cross-link with the DNA of *E. coli* and eradicated the persister cells. In another study, Sun et al. screened the antibacterial activity for 5170 FDA-approved drugs and noticed that zidovudine, an anti-HIV agent (inhibits the transcription), represented an antibacterial activity against multidrug-resistant *K. pneumoniae* strains, with MICs ranging from 0.1 to 6.5 μM. Federica et al. repurposed the gallium nitrate, which was used for the hypercalcemia of malignancy, as an antibiofilm agent against *A. baumannii*.[50] A 3.5-fold decrease of biofilm was found under 32 μM of gallium nitrate, and the biofilm was barely observed when treated with 64 μM of gallium nitrate. Daniel-Hoffmann et al. first revealed the antimicrobial activity of AS101 against ESBL-producing *K. pneumoniae*, with minimum inhibitory concentrations (MICs) ranging from 9 to 18 μg/ml, and in another study, they also found the bactericidal activity of AS101 against *Enterobacter cloacae*, with a MIC of 9.4 μg/ml.[51,52] AS101 also showed promising potential against carbapenem-resistant *K. pneumoniae* (CRKP) and carbapenem-resistant *A. baumannii* (CRAB). Our previous studies,[14,15] involving CRKP and CRAB clinical isolates with different antibiotic-resistant profiles and carbapenem-resistant mechanisms, reported that AS101 exhibits antibacterial activities against these drug-resistant pathogens, with MIC ranges of both <0.5–32 μg/ml for CRKP and CRAB. In the current work, AS101, previously an immunomodulatory agent,[53] demonstrated a remarkably antibacterial effect on CPEC, with MICs ranging from <0.5 to 32 μg/ml far below its 50% cytotoxicity (145 μg/ml).[11]

EDTA has been reported to chelate the cations which were needed to maintain the stability of bacterial outer membrane and to increase the membrane permeability.[54] Smith et al. optimized the arylomycin and generated G0775 targeting LepB, an enzyme inside bacteria.[54] In their study, 4 mM of EDTA was added to increase the membrane permeability of *E. coli* and further, led to a decrease of G0775 MIC (64 folds). Our result showed a 32-fold decrease of AS101 MIC against CRE-415 with the addition of 4 mM EDTA (Table 2), speculating a target inside the bacterial cell. 320 mM of mannitol was employed as a ROS scavenger,[26] and AS101 MIC against CRE-415 was observed to increase by 16 times after co-cultured with the mannitol (Table 2), suggesting that ROS may play a role in the antibacterial activity of AS101 against *E. coli*. Agree with the finding of the pharmaceutical manipulations, the cellular ROS level in CRE-415 raised in a dose-dependent manner when treated with AS101 (Fig. 3). Furthermore, ROS has been documented to damage bacterial DNA.[55] In this study, the DNA of CRE-415 was observed to be fragmented in a dose-dependent manner while treated with different concentrations of AS101 (Fig. 4). The mechanistic investigations suggest that AS101 would enter the bacterial cell, and induce ROS generation, leading to DNA fragmentation.

Although colistin has been considered the last-resort antibiotic against carbapenem-resistant crises,[56] instances of failed-to-treat infections by carbapenem-resistant crises have been reported.[57,58] In a previous study, Wang et al. examined the *in vivo* treatment effects of colistin against *E. coli*.[57] A urinary tract infection mouse model was established via a uropathogenic *E. coli* strain, and the infected mice were treated with six dosing regimens of colistimethate sodium (CMS), from 11.7 mg/kg q12h to 93.6 mg/kg q24h. The bacterial loads in urine, bladder, and kidney were determined. Even though significant decreases were observed for all dosing regimens, the results of bacterial loads varied, implying an unsteady treatment effect of colistin against *E. coli*. In this study, we observed a similar result for colistin and that AS101 possessed a more steady and remarkable therapeutic effect against *E. coli* CRE-415, compared to colistin where the standard deviations were large (Table 3).

Agreed with our previous studies,[14,15] AS101 demonstrated antibacterial activities against CPEC isolates via cracking the bacterial surfaces, entering bacterial cells, and inducing ROS damage. A further mechanistic study

unveiled that the accumulated ROS caused by AS101 could damage bacterial DNA. The *in vivo* study also revealed that AS101 possessed a steady treatment effect in a sepsis mouse model. Our findings provided further evidence for the antibiotic development of AS101.

## Declaration of competing interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.07.002.