

Small Molecule Adjuvants Potentiate Colistin Activity and Attenuate Resistance Development in *Escherichia coli* by Affecting *pmrAB* System

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Background: Colistin is one of the last-resort antibiotics to treat multi-drug resistant (MDR) Gram-negative bacterial infections in humans. Further, colistin has been also used to prevent and treat Enterobacteriaceae infections in food animals. However, chromosomal mutations and mobile colistin resistance (*mcr*) genes, which confer resistance to colistin, have been detected in bacterial isolates from food animals and humans worldwide; thus, limiting the use of colistin. Therefore, strategies that could aid in ameliorating colistin resistance are critically needed.

Objective: Investigate the adjuvant potential of novel small molecules (SMs) on colistin.

Materials and Methods: Previously, we identified 11 membrane-affecting SMs with bactericidal activity against avian pathogenic *Escherichia coli* (APEC). Here, we investigated the potentiation effect of those SMs on colistin using checkerboard assays and wax moth (*Galleria mellonella*) larval model. The impact of the SM combination on colistin resistance evolution was also investigated by analyzing whole genome sequences of APEC isolates passaged with colistin alone or in combination with SMs followed by quantitating *pmrCAB* and *pmrH* expression in those isolates.

Results: The SM combination synergistically reduced the minimum bactericidal concentration of colistin by at least 10-fold. In larvae, the SM combination increased the efficacy of colistin by two-fold with enhanced (>50%) survival and reduced (>4 logs) APEC load. Further, the SM combination decreased the frequency (5/6 to 1/6) of colistin resistance evolution and downregulated the *pmrCAB* and *pmrH* expression. Previously unknown mutations in *pmrB* (L14Q, T92P) and *pmrA* (A80V), which were predicted deleterious, were identified in the colistin-resistant (Col^R) APEC isolates when passaged with colistin alone but not in combination with SMs. Our study also identified mutations in hypothetical and several phage-related proteins in Col^R APEC isolates in concurrent with *pmrAB* mutations.

Conclusion: Our study identified two SMs (SM2 and SM3) that potentiated the colistin activity and attenuated the development of colistin resistance in APEC. These SMs can be developed as anti-evolution drugs that can slow down colistin resistance development.

Keywords: colistin, small molecules, *pmrCAB*, *pmrH*, resistance, anti-evolution drug

Introduction

Colistin, a cyclic polypeptide antibiotic isolated in 1947 from soil bacterium *Bacillus colistinus*, is active against many Gram-negative bacteria.^{1,2} It binds to lipopolysaccharide (LPS) and phospholipids in the outer cell membrane of Gram-negative bacteria and competitively displaces divalent cations from the phosphate

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groups of membrane lipids, which leads to disruption of the outer cell membrane, leakage of intracellular contents, and bacterial death.³ In particular, it is used as a last-resort antibiotic for the treatment of severe infections in humans caused by multi-drug resistant (MDR) Gram-negative pathogens such as *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*.^{2,4} Further, it has been used in veterinary medicine for decades, mainly as a growth promoter (feed additive) or for the prevention and treatment of Enterobacteriaceae infections, including avian pathogenic *E. coli* (APEC) in poultry.^{1,2,4,6} Colistin usage in food animals is widespread, particularly high in low- and middle-income countries, and has been increasing since the mid-twentieth century.^{1,4} A 13% increase in colistin usage has been reported in Brazil, Russia, India, China, and South Africa from 2000 to 2010.⁴ In China (the largest user of colistin), 2470–2875 metric tons of colistin was used to treat *E. coli* and *Salmonella* infections in chicken and pig farms from 2011 to 2015.⁴ In the European Union, colistin was the fifth most widely used antibiotic during 2013–2015 to treat and prevent infectious diseases in food animals.^{2,4} In the USA, colistin (First Guard™ sterile powder; colistimethate sodium) is approved by the Food and Drug Administration (FDA) for the treatment of APEC infection in day-old chicks (Code of Federal Regulations 21 Section 522.468). In the Netherlands, colistin was the most predominant antibiotic used to treat APEC induced *E. coli* peritonitis syndrome (EPS) in layer flocks in 2013.⁵ However, with the use of colistin, bacterial resistance to colistin has been reported worldwide.^{1,7} Consequently, many countries in the world, including USA, European Union, and China, have now banned the growth promotion and prevention uses of colistin in food animals.^{1,4}

Colistin resistance is typically mediated by chromosomal mutations in two-component systems, such as PmrAB and PhoPQ,⁷ or by horizontal transfer of plasmid carrying mobile colistin resistance (*mcr*) genes.⁸ Multiple chromosomal mutations (most commonly, *pmrB*, *pmrA*, *mgrB*, *phoP*, and *phoQ*) and *mcr* genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, *mcr-8*, and *mcr-9*) have been detected in bacterial isolates, including *E. coli*, from food animals (pig, cattle, poultry) and humans worldwide; thus, threatening the use of this last-resort antibiotic.^{1,4,7,9,11} The chromosomal mutations modify the lipid A moiety of LPS, which is a primary target of colistin, and thereby leads to colistin resistance.⁷ Specifically, PmrAB and PhoPQ mutations induce the transcription of *pmrCAB* and

pmrHFIJKLM (or *arnBCADTEF*) operons, which leads to the synthesis of phosphoethanolamine (PETN) and 4-amino-4-deoxy-L-arabinose (L-ara4N), respectively, and then subsequently transfer it to lipid A.⁷ The addition of PETN or L-ara4N reduces the negative charge of lipid A and thereby decreases colistin binding with its target which results in reduced efficacy of colistin.⁷ Notably, the occurrence of colistin resistance is reported more frequently in food animals (0.9–76.9%) than humans (0.1–8.8%),^{4,11} which is in accordance with the higher selection pressure posed by the heavy use of colistin in food animals.^{4,11} Therefore, strategies to decrease colistin use and ameliorate colistin resistance are critically needed to preserve the future use of colistin in treating MDR pathogens.¹²

One potential approach to ameliorate colistin resistance is the use of antibiotic adjuvants (or potentiators) which can increase the efficacy of colistin, ultimately requiring lower doses of colistin for treatments.^{13,14} In previous studies, 2-aminoimidazoles (2-AIs),¹³ (*E*)-2--hexenal,¹⁵ indole,¹⁵ N-acetylcysteine,¹⁶ resveratrol,¹⁷ niclosamide,¹⁸ and pentamidine¹⁹ were identified as colistin adjuvants against different Gram-negative pathogens. Importantly, colistin adjuvants can also reverse the colistin resistance so that the colistin is effective against the previously resistant bacteria.^{13,18,20} Specifically, adjuvants can abrogate or reduce the extent of lipid A modification by reducing the expression of *mcr* and *pmrAB/phoPQ* genes;^{13,18,19,21,22} thus, overcoming the colistin resistance. In this study, we investigated the adjuvant potential of previously identified membrane-acting small molecules (SMs)²³ with colistin against APEC. Because of the membrane-affecting properties of these SMs,²³ similar to previously identified colistin adjuvants which have been shown to potentiate the colistin effect by disrupting as well as permeabilizing the bacterial membrane,^{19,21,24,27} these SMs can work synergistically with colistin, which is also a membrane-active antibiotic;² thereby, augmenting the colistin mediated disruption of the bacterial membrane.

APEC, an extra-intestinal pathogenic *E. coli* (ExPEC) and a model pathogen of this study, is the most common bacterial pathogen of poultry.²⁸ In the USA, it is estimated that at least 30% of commercial flocks are affected by APEC, at any point in time.²⁸ In poultry, APEC causes a wide range of localized and systemic infections; commonly referred to as avian colibacillosis.²⁹ Colibacillosis results in significant morbidity and mortality of chickens, which translates into multi-million dollar annual losses for

all facets of the world's poultry industry.²⁸ Further, recent studies have reported APEC as a foodborne human uropathogen and commonalities in diseases caused by ExPEC strains in humans.^{28,30} APEC is also considered as a source of antibiotic-resistant genes (ARGs) to human pathogens.³¹ Therefore, effective control of APEC is beneficial to both human and animal health by reducing the foodborne transmission of ExPECs and ARGs.²⁸

Our study identified two SMs (SM2 and SM3) that potentiated the colistin activity and attenuated the development of colistin resistance in APEC. The SM combination also downregulated the expression of *pmrCAB* and *pmrH* genes responsible for colistin resistance. Previously unknown mutations in *pmrB* and *pmrA* genes were identified. Mutations were also identified in other genes encoding hypothetical proteins and several phage-related proteins in concurrent with *pmrAB* mutations, which can help in better understanding of molecular sequelae associated with colistin resistance. These SMs can be developed as anti-evolution drugs that can slow down colistin-resistance development.³² Although our study tested colistin adjuvant potential of SMs only against APEC strains, future studies on the adjuvant potential of these SMs with colistin against human ExPECs and other Gram-negative pathogens can have significant implication in treating MDR pathogens.

Materials and Methods

Bacterial Strains, Culture Conditions and Media

Luria-Bertani (LB) broth (BD Difco™) was used for routine propagation of APEC O78, O1, and O2. These APEC isolates were isolated from chickens (O1 – lung, accession number: CP000468; O2 – airsac, accession number: CP006834) and turkey (O78 – lung, accession number: CP004009) clinically diagnosed with various forms of colibacillosis and serotyped using O-serotyping slide agglutination test. M63 minimal media was used in the checkerboard assay as these SMs were identified as growth inhibitors in M63 media.²³ Rifampicin resistant (Rif^r) APEC O78 generated through spontaneous mutation in our previous study²³ was used for the wax moth larvae study.

Small Molecules and Antibiotics

All the SMs (SM 1–11) used in this study were obtained from ChemBridge™, dissolved in 100% dimethyl sulfoxide (DMSO) at 100 mM, and stored at –80°C as described

previously.²³ These SMs were identified as APEC growth inhibitors in our earlier study²³ through screening of pre-selected enriched SM library containing 4182 SMs. Moreover, this SM library was assembled through pre-screening of ~81,000 compounds.³³ These SMs possess pyrrolidinyll (SM2, SM3, and SM7), imidazole (SM4, SM5, and SM6), piperidine (SM1 and SM11), quinoline (SM8 and SM9), and nitrophenyl (SM10) scaffolds.²³ All the antibiotics used in this study (colistin sulfate salt, ciprofloxacin, and tetracycline) were purchased from Sigma-Aldrich. The selected antibiotics belong to different classes of antibacterials with different mechanisms of action and are currently being used in the poultry industry.²³ Antibiotic stocks were prepared following CLSI guidelines (M100, 28th edition).³⁴

MIC and MBC Determination

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of SMs against APEC serotypes (O78, O1, and O2) were identified in our earlier study.²³ MIC and MBC of antibiotics against APEC serotypes were determined by twofold serial dilution in M63 media (Table S1 and S2). Briefly, twofold serially diluted antibiotics were added to 100 µL of 0.05 OD₆₀₀ adjusted APEC culture and incubated at 37°C for 12 h in Sunrise-Absorbance microplate reader (Tecan Group Ltd. San Jose, CA) with kinetic turbidity measurement set for every 30 min. MIC was indicated by the lowest concentration of antibiotic with non-elevated OD₆₀₀; whereas MBC was determined by plating the antibiotic-treated culture on the LB agar plate. Two independent experiments were conducted.

Checkerboard Assay

The synergy potential of SMs with antibiotics was assessed using a checkerboard assay as previously described.^{35,36} Briefly, SMs were diluted (1×, 0.8×, 0.6×, 0.4×, 0.2×, and 0.1× MBC) along the abscissa, whereas; antibiotics were diluted along the ordinate (1×, 0.8×, 0.6×, 0.4×, 0.2×, and 0.1× MBC) in 100 µL of 0.05 OD₆₀₀ adjusted APEC O78 culture in a 96-well plate. We tested MBC in this assay because these SMs were found bactericidal against APEC in our earlier study²³ and also MBC can provide more reliable and quantitative data of SM-antibiotic combination as compared to MIC. After the addition of SMs and antibiotics, the plate was incubated at 37°C for 12 h in Sunrise-Absorbance microplate reader and MBC of SM-antibiotic combination was determined as above. The fractional

inhibitory concentrations (FICs) of SMs in combination with antibiotics were determined as described previously.³⁷ Synergistic, indifferent, and antagonistic activities were defined by FICs of 0.5, 0.51 to 4.0, and >4.0, respectively. Four SMs (SM2, SM3, SM5, and SM7) that showed synergism with most of the antibiotics tested were selected for further study. However, SM5 belongs to the imidazole group and the potentiating activity of imidazoles on colistin has been already reported,¹³ therefore, SM5 was not studied further. On the other hand, SM7, which possesses a pyrrolidinyl moiety similar to SM2 and SM3, was not studied due to its unavailability in the ChemBridge library. Therefore, two SMs (SM2 and SM3) which were synergistic with most of the antibiotics tested and possessed novel scaffold (pyrrolidinyl) with no previous report of potentiation effect with colistin were selected for further studies. For the selected SMs, the checkerboard assay was also conducted against APEC serotypes O1 and O2 as above.

In vivo Synergy Studies in Wax Moth Larva

The in vivo synergy potential of SMs (SM2 and SM3) with antibiotics was determined in the wax moth (*G. mellonella*) larva model as described previously.³⁸ SMs (12.5 µg) were combined with antibiotics: colistin 0.3125 mg/kg; tetracycline 1.5625 mg/kg and ciprofloxacin 2.5 mg/kg. The antibiotic dose (the dose that provides minimal protection to the larva against APEC infection) was selected based on the preliminary study (Table S3). Briefly, larvae (n=15 larva/group) were infected with 6.4×10^4 CFU of Rif^r APEC O78 as described previously²³ through the right hind pro-leg using an insulin syringe. Larvae were then treated with colistin (0.3125 mg/kg), a combination of colistin and SMs (0.3125 mg/kg+12.5 µg), tetracycline (1.5625 mg/kg), and a combination of tetracycline and SMs (1.5625 mg/kg+12.5 µg) within 30 min of infection as described previously;^{38,39} or after 2 h of infection for ciprofloxacin (2.5 mg/kg) and the combination of ciprofloxacin and SMs (2.5 mg/kg+12.5 µg), as described previously.⁴⁰ Uninfected larvae and infected larvae treated with buffer mix (DMSO-15% + PBS) were used as controls. Larval survival was monitored every 12 h for 3 days. Dead larvae were collected at each time point and intra-larval APEC load was quantified as described previously²³ by plating the homogenized larval suspension on MacConkey agar plate supplemented with 50 µg/mL rifampicin. APEC load in live larvae was quantified as described above after 72 h post-infection. For the survival

analysis, Kaplan-Meier survival curves were generated and statistically analyzed between the treated groups using Log-rank test ($P < 0.05$). The APEC load was statistically compared between the treated groups using a one-way analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$). Two independent experiments were conducted.

Impact of SM Combination on Colistin-Resistance Evolution

The impact of SMs (SM2 and SM3) on in vitro colistin-resistance evolution was analyzed using the sub-inhibitory concentration of SMs as described previously.⁴¹ Briefly, six parallel independent APEC O78 cultures (DMSO 1–6, Col 1–6, Col + SM2 1–6, and Col + SM3 1–6) were passaged in M63 media starting with the sub-inhibitory concentration of colistin (0.0625 µg/mL) and in the presence or absence of $0.5 \times$ MIC (SM2: 50 µM; SM3: 50 µM) of SMs. Cultures were allowed to grow for 24 h, and 20 µL of grown cultures were transferred to new wells containing fresh M63 media and increasing concentration of colistin (0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 µg/mL) up to 8 µg/mL (ie, beyond resistance breakpoint). SMs were added at each passage at $0.5 \times$ MIC concentrations. APEC cultures containing no colistin and no SM (1% DMSO; DMSO 1–6) were used as controls and passaged similarly (8 passages) to cultures containing colistin or colistin and SMs. The growth of cultures was monitored at each passage and only the grown cultures were selected for further passage. For the non-grown cultures (ie, cultures not grown until the last colistin passage), glycerol stock was made from the previous passage by regrowing the remnant (80 µL) in fresh M63 media containing respective colistin and SM concentrations and stored at -80°C for further analysis. At the end of the experiment, the MIC of colistin for both grown (Col-1, Col-3, Col-4, Col-5, Col-6, Col + SM2-2 and Col + SM3-5) and non-grown (Col-2, Col + SM2-1, Col + SM2-3, Col + SM2-4, Col + SM2-5, Col + SM2-6, Col + SM3-1, Col + SM3-2, Col + SM3-3, Col + SM2-4, and Col + SM3-6) cultures was determined as described above. The selected grown and non-grown cultures (Col-1, Col-3, Col-5, Col-6, Col + SM2-2, Col + SM2-4, Col + SM2-6, Col + SM3-2, Col + SM3-5, and Col + SM3-6), including control cultures (DMSO-1 and DMSO-3), were plated on M63 agar plates and single colonies were isolated. Cultures were selected from each group including both colistin-resistant (Col^R; Col-1, Col-3, Col-5, Col-6, Col + SM2-2, and Col + SM3-5) and susceptible (Col^S; Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col

+ SM3-6) phenotypes. Control susceptible isolates (DMSO-1 and DMSO-3) were also selected to provide a baseline for genomic analysis. The isolated colonies were grown overnight, DNA was extracted using MasterPure™ DNA Purification Kit (Epicentre) and the whole genome was sequenced.

Whole Genome Sequencing

The Whole genome sequencing (WGS) of APEC isolates (DMSO-1, DMSO-3, Col-1, Col-3, Col-5, Col-6, Col + SM2-2, Col + SM2-4, Col + SM2-6, Col + SM3-2, Col + SM3-5, and Col + SM3-6) was performed on an Illumina MiSeq platform. Briefly, an initial concentration of 0.3 ng/μL DNA was used for library preparation using the Nextera XT Library preparation kit (Illumina Inc.). After dual indexing, the library was bead normalized, and the samples were pooled at equal volume and sequenced using 2×300 paired-end V3 chemistry.

Mutations and Variants Analysis

The chromosomal mutations pertaining to colistin resistance in the genome of APEC isolates (DMSO-1, DMSO-3, Col-1, Col-3, Col-5, Col-6, Col + SM2-2, Col + SM2-4, Col + SM2-6, Col + SM3-2, Col + SM3-5, and Col + SM3-6) were initially searched using PointFinder software 3.1.0 (<https://bitbucket.org/genomicpidemiology/pointfinder/src/master/>) as described previously.⁴² Briefly, the quality of the sequence reads was determined using FastQC (version 0.11.8) package (Babraham Bioinformatics). The sequences were then assembled using the SPAdes (version 3.9.0) package. The chromosomal mutations in the assembled genome were identified using the PointFinder database (https://bitbucket.org/genomicpidemiology/pointfinder_db/src/master/). The mutations present in Col^R isolates (Col-1, Col-3, Col-5, Col-6, Col + SM2-2, and Col + SM3-5) were compared with Col^S isolates (Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col + SM3-6), including control isolates (DMSO-1 and DMSO-3), to identify the unique mutations responsible for colistin resistance. For a further detailed analysis of mutations and variants, read mapping and variants calling method as implemented in CLC Genomics Workbench was used. The variant detection tools in CLC Genomics Workbench implemented is based on the Neighborhood Quality Standard (NQS) algorithm.^{43,44} Demultiplexing of the data was performed using the Casava pipeline. Further analysis of de-multiplexed sequence data as FASTQ format was performed in CLC Genomics workbench v.12.0. Paired reads were taken through a quality check followed by a trimming step to ensure a minimum average

PHRED score of 30. APEC O78 complete genome (GenBank Accession: CP004009.1) was used as a reference for read mapping and annotation. Reads were mapped against the reference genome using local alignment to determine structural variants such as insertions, deletions, inversions, translocations, and tandem duplications in the experimental isolates. This information was used to improve the alignment of the reads in an existing read mapping. Variants were called when there is a minimum coverage of 10× and a minimum frequency of 20%. To avoid false positives, we removed low-quality variants that have an average base quality of less than 20 and forward and reverse read balance of less than 0.2. Variants were then annotated using the coding sequence (CDS) and gene annotation information from the reference genome. Relevant known genomic variants in the Col^R isolates were tested for the presence or absence in Col^S isolates by comparing the variants in the Col^R isolate with the read mappings of Col^S isolates, including control isolates. Specific variants that are present only in the Col^R isolates and not in Col^S isolates, control isolates, and reference genome were taken into account. These variants were then screened for their presence in the CDS as well as for amino acid (AA) change. The complete sequence data used for the above analysis have been deposited in the NCBI sequence read archive (SRA) under the Bioproject number PRJNA591010 (URL: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA591010?reviewer=capd9kv55kk447p5if9ucfal0>).

pmrCAB and *pmrH* Expression Analysis

To corroborate the findings of mutation analysis, relative expression of *pmrCAB* and *pmrH* in APEC isolates (DMSO-1, DMSO-3, Col-1, Col-3, Col-5, Col-6, Col + SM2-2, Col + SM2-4, Col + SM2-6, Col + SM3-2, Col + SM3-5, and Col + SM3-6) was quantitated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as described previously.^{45,46} APEC isolates frozen in glycerol at −80°C were grown overnight at 37°C with shaking at 200 rpm and total RNA was extracted using RNeasy mini kit (Qiagen) following the manufacturer's instructions. RNA quantity and quality were measured using nanodrop 2000c spectrophotometer and 1% agarose gel electrophoresis. DNA traces were removed using the genomic DNA elimination mix (Qiagen). Approximately 5 μg of purified RNA was used to synthesize cDNA using RT² First Strand Kit (Qiagen). The RT-qPCR was performed using Maxima SYBR Green/ROX qPCR master mix (Thermo Fisher Scientific) following the manufacturer's instructions in a RealPlex² Mastercycler® (Eppendorf) with

55°C annealing temperature. The primers (Table S4) were designed using PrimerQuest Tool and obtained from Integrated DNA Technologies (IDT). The data were normalized to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relative fold change in expression was calculated in each APEC isolate (Col-1, Col-3, Col-5, Col-6, Col + SM2-2, Col + SM2-4, Col + SM2-6, Col + SM3-2, Col + SM3-5, and Col + SM3-6) by comparing with control isolates (DMSO-1 and DMSO-3) using the $\Delta\Delta C_t$ method.⁴⁷ The data were statistically analyzed using the Student's *t*-test ($P < 0.05$). Two-independent experiments were conducted.

Results

Small Molecules Exhibited Synergistic Interaction with Colistin in vitro

Among the 11 SMs tested in combination with antibiotics (colistin, tetracycline, and ciprofloxacin) in checkerboard assay, four SMs (SM2, SM3, SM5, and SM7) reduced the MBC of all three antibiotics by at least 10-fold (Table 1). Interestingly, most of the SMs (SM2, SM3, SM4, SM5, SM6, SM7, SM9, and SM11) reduced 10-fold MBC (2 µg/mL to 0.2 µg/mL) of colistin (Table 1), whereas four SMs (SM2, SM3, SM5, and SM7) reduced 10-fold MBC (128 µg/mL to 12.8 µg/mL) of tetracycline (128 µg/mL to 12.8 µg/mL) (Table 1) and eight SMs (SM2, SM3, SM4, SM5, SM6, SM7, SM9, and SM11) reduced 10-fold MBC (1 µg/mL to 0.1 µg/mL) of ciprofloxacin (Table 1). The MBCs of SMs upon the combination with antibiotics are displayed in Table 2. The combination of colistin also reduced the MBC of all SMs by at least 10-fold. Tetracycline combination reduced the 10-fold MBC of SM3 and SM7, whereas the ciprofloxacin combination reduced only the MBC of SM4 by 10-fold.

Among the 11 SMs tested, SM5 showed synergistic interaction (FIC=0.2–0.5) with all three antibiotics tested

(Table S5). Interestingly, most of the SMs (SM2, SM3, SM4, SM5, SM6, SM7, SM8, SM9, and SM11) showed synergistic interaction (FIC=0.2) with colistin. Four SMs (SM2, SM3, SM5, and SM7) showed synergism (FIC=0.2–0.5) with tetracycline, and three SMs (SM4, SM5, and SM11; FIC=0.2–0.5) with ciprofloxacin. Four SMs (SM2, SM3, SM5, and SM7) that displayed synergism with most of the antibiotics tested were selected for further study. However, SM5 belongs to the imidazole group and the colistin potentiating activity of imidazoles has been already reported;¹³ therefore, SM5 was not studied further. SM7, which possess pyrrolidinyl moiety similar to SM2 and SM3, was not studied further due to unavailability in the ChemBridge library.

The selected SMs (SM2 and SM3) also reduced the MBC of colistin by at least 10-fold (4 µg/mL to 0.4 µg/mL) against APEC O1 and O2, similar to O78, and also showed a synergistic interaction with colistin (Table 3).

Small Molecule Combination Increased the Efficacy of Colistin in Wax Moth Larvae

Two selected SMs (SM2 and SM3; 12.5 µg), when combined with colistin, increased (SM2-46.67% and SM3-57.14%) the survival of larvae (Figure 1A) as well as significantly ($P < 0.001$) reduced the intra-larval APEC O78 load (SM2-4.82 logs and SM3-5.24 logs) as compared to colistin treatment alone (Figure 1B). Similarly, these SMs increased (SM2-13.34%) the survival of larvae and reduced the intra-larval APEC O78 load (SM2-0.82 logs and SM3-0.54 logs) when combined with ciprofloxacin as compared to ciprofloxacin treatment alone (Figure S1A and B). However, no difference in the larval survival and intra-larval APEC O78 load was observed when these SMs were combined with tetracycline (Figure S1C and D).

Table 1 Minimum Bactericidal Concentration (MBC) of Antibiotics When Tested in Combination with SMs

AB	MBC (µg/mL)											
	AB Alone	AB + SM										
		SM1	SM2	SM3	SM4	SM5	SM6	SM7	SM8	SM9	SM10	SM11
Col	2	1.6 ^I	0.2 ^S	0.2 ^S	0.2 ^S	0.2 ^S	0.2 ^S	0.2 ^S	0.8 ^S	0.2 ^S	0.8 ^I	0.2 ^S
Tet	128	76.8 ^I	12.8 ^S	12.8 ^S	128 ^I	12.8 ^S	76.8 ^I	12.8 ^S	128 ^I	51.2 ^I	128 ^I	25.6 ^I
Cip	1	1 ^I	0.1 ^I	0.1 ^I	0.1 ^S	0.1 ^S	0.1 ^I	0.1 ^I	0.2 ^I	0.1 ^I	1 ^I	0.1 ^S

Notes: ^SSynergistic, ^IIndifferent.

Abbreviations: AB, antibiotic; Col, colistin; Tet, tetracycline; Cip, ciprofloxacin.

Table 2 Minimum Bactericidal Concentration (MBC) of SMs When Tested with Antibiotics

	MBC (μM)			
	SM Alone	SM + Col	SM + Tet	SM + Cip
SM1	150	15	60	150
SM2	100	10	40	60
SM3	100	10	10	100
SM4	50	5	50	5
SM5	15	1.5	6	6
SM6	15	1.5	9	12
SM7	50	5	5	30
SM8	15	1.5	15	15
SM9	30	3	12	24
SM10	50	20	50	50
SM11	100	10	40	40

Abbreviations: Col, colistin; Tet, tetracycline; Cip, ciprofloxacin.

Small Molecule Combination Decreased the Frequency of Colistin-Resistance Evolution

The experimental evolution of colistin resistance in the presence ($0.5\times$ MIC) or absence of SMs (SM2 and SM3) was assessed through successive passages of APEC cultures in increasing concentrations of colistin (starting with sub-inhibitory concentration; $0.0625\ \mu\text{g/mL}$). Most of the APEC cultures (5/6; Col-1, Col-3, Col-4, Col-5, and Col-6) passaged only in the presence of colistin were found resistant, whereas only 1 culture (1/6; Col + SM2-2 and Col + SM3-5) was found resistant to colistin when combined with SM2 and SM3 (Figure 2A). Four of the APEC cultures (Col-1, Col-4, Col-5, and Col-6) passaged only in presence of colistin had MIC $64\ \mu\text{g/mL}$ and the other two APEC cultures had MIC $32\ \mu\text{g/mL}$ (Col-3) and $4\ \mu\text{g/mL}$ (Col-2). On the other hand, only one APEC culture had MIC $64\ \mu\text{g/mL}$ (Col + SM2-2 and Col + SM3-5) and other cultures (Col + SM2-1,

Col + SM2-3, Col + SM2-4, Col + SM2-5, Col + SM2-6, Col + SM3-1, Col + SM3-2, Col + SM3-3, Col + SM3-4, and Col + SM3-6) had MIC $\leq 4\ \mu\text{g/mL}$ when colistin was combined with SM2 and SM3. Most of these cultures (Col + SM2-1, Col + SM2-4, Col + SM2-5, Col + SM2-6, Col + SM3-2, Col + SM3-3, and Col + SM2-4) had MIC $2\ \mu\text{g/mL}$; whereas few (Col + SM2-3, Col + SM3-1, and Col + SM3-6) had MIC $4\ \mu\text{g/mL}$. The chemical structure and ChemBridge ID of these two SMs are displayed in Figure 2B, Table S6.

Previously Unknown Mutations in *pmrB* and *pmrA* Genes Were Identified in Col^R APEC Isolates

The PointFinder database mediated comparative analysis of chromosomal mutations revealed point mutations in *pmrA* and *pmrB* genes in Col^R isolates (Col-1, Col-3, Col-5, Col-6, Col + SM2-2, and Col + SM3-5) as compared to Col^S (Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col + SM3-6) and control isolates (DMSO-1 and DMSO-3) (Table 4). The most common mutation was in *pmrB* p.L14Q (Col-5, Col-6, Col + SM2-2, and Col + SM3-5), where thymine was replaced by adenine (CTG to CAG) resulting in glutamine instead of leucine (L to Q). The mutation was also observed in *pmrB* p.T92P (Col-3), where adenine was replaced by cytosine (ACC to CCC) resulting in proline instead of threonine (T to P). Similarly, in *pmrA* p.A80V (Col-1), cytosine was replaced by thymine (GCT to GTT) resulting in valine instead of alanine (A to V).

Consistent with the decreased frequency of colistin-resistance evolution, no *pmrA* and *pmrB* mutations were present in most of the sequenced APEC isolates (4/6) which were passaged with colistin and SM combination (absent in Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col + SM3-6; present only in Col + SM2-2 and Col + SM3-5) as compared to colistin alone (present in Col-1, Col-3, Col-5, and Col-6) passaged APEC isolates (Figure 3). Similar to results obtained using the PointFinder database, in reads mapping and variants calling analysis, non-synonymous mutations were observed in Col^R isolates in genes encoding sensor protein BasS/PmrB and two-component response regulator BasR/PmrA (Figure 3, Table 5). Single nucleotide variation (SNV; A to T) was observed in gene encoding sensor protein BasS/PmrB in most (4/6; Col-5, Col-6, Col + SM2-2, and Col + SM3-5) of the Col^R isolates, compared to Col^S isolates which resulted in glutamine (Q) instead of leucine (L) at position 14 of PmrB protein

Table 3 Minimum Bactericidal Concentration (MBC) of Colistin Against APEC O1 and O2 When Tested with Selected SMs

	MBC (μg/mL)					
	APEC O1			APEC O2		
	Col Alone	Col + SM	FIC	Col alone	Col + SM	FIC
SM2	4	0.4	0.2 ^S	4	0.4	0.2 ^S
SM3	4	0.4	0.2 ^S	4	0.4	0.2 ^S

Note: ^SSynergistic.

Abbreviation: Col, colistin.

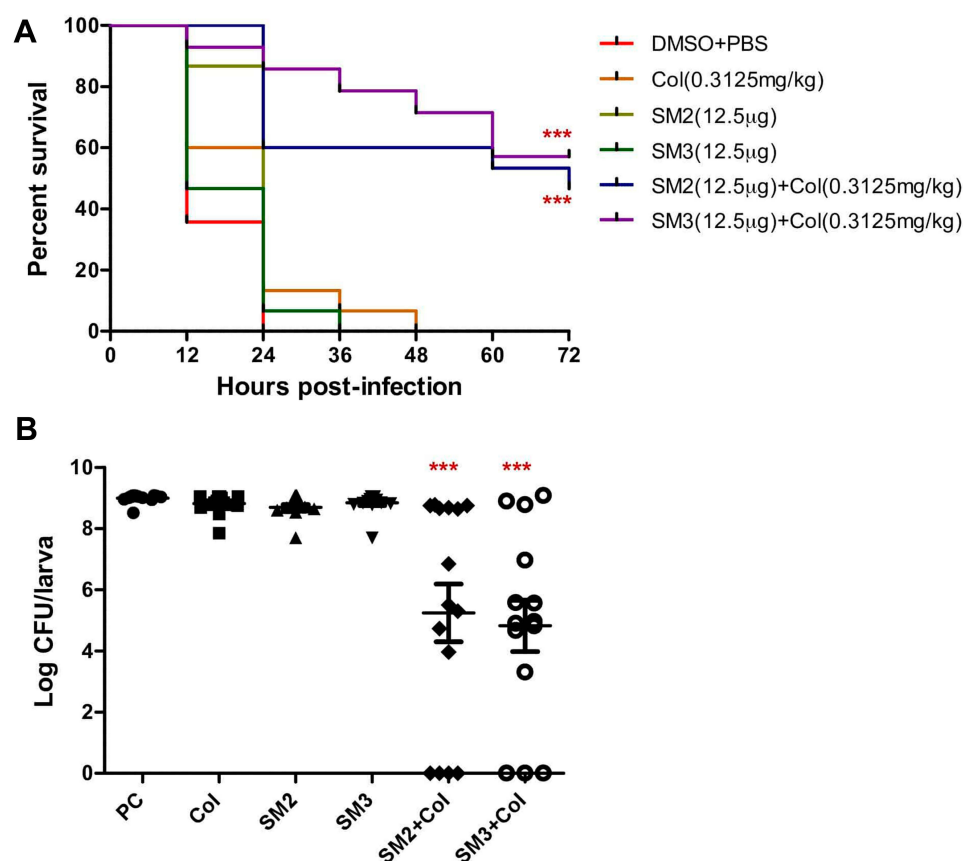


Figure 1 (A) Survival curve of wax moth larvae treated with colistin alone or in combination with SMs. Larvae ($n=15$ larva/group) were infected with 6.4×10^4 CFU of Rif^R APEC O78 and then treated with colistin (0.3125 mg/kg) and combination of colistin and SMs (0.3125 mg/kg+12.5 μg) within 30 min of infection. Larval survival was monitored every 12 h for 3 days. (B) APEC load inside wax moth larvae treated with colistin alone or in combination with SMs. APEC load in larvae was quantified by plating the homogenized larval suspension on MacConkey agar plates supplemented with 50 μg/mL rifampicin. PC-infected and buffer mix (DMSO+PBS) treated larvae, *** $P<0.0001$.

(*pmrB* p. L14Q). In the same gene, SNV (T to G) was observed in isolate Col-3 which resulted in proline (P) instead of threonine (T) at position 92 (*pmrB* p. T92P). Additionally, SNV (G to A) was also observed in gene encoding two-component response regulator BasR/PmrA in one of the Col^R isolates (Col-1), which resulted in valine (V) instead of alanine (A) at position 80 of PmrA protein (*pmrA* p. A80V).

Small Molecule Combination Downregulated the *pmrCAB* and *pmrH* Expression

Colistin resistance in bacteria is mediated by the upregulation of genes of *pmrCAB* and *pmrHFIJKLM* operons;^{20,45,48} therefore, suppressing the expression of involved genes can prevent the evolution of colistin resistance. Specifically, the upregulated *pmrC* and *pmrH* expression results in lipid A modifications; thereby, subsequently decreases the binding and activity of colistin against bacteria.⁷ Consistent with the

absence of *pmrA* and *pmrB* mutations in most of the APEC isolates passaged with colistin and SM combination, the level of *pmrCAB* and *pmrH* expression was lower in most of the APEC isolates passaged with colistin in combination with SMs (Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col + SM3-6) as compared to APEC isolates (Col-1, Col-3, Col-5, and Col-6) passaged alone with colistin (Figure 4A and B). The level of *pmrCAB* and *pmrH* expression in each APEC isolate is displayed in Figure 4A and B. Overall, the expression of *pmrCAB* and *pmrH* expression was lower in Col^S isolates (Col + SM2-4, Col + SM2-6, Col + SM3-2, and Col + SM3-6) as compared to Col^R (Col-1, Col-3, Col-5, Col-6, Col + SM2-2, and Col + SM3-5) isolates. Further, as compared to control APEC isolates (DMSO-1, DMSO-3), the expression level (on average) of *pmrC* was 17.9 ± 3.8 -, 6.4 ± 0.3 -, and 3.8 ± 0.5 -fold higher in APEC isolates passaged with colistin, colistin and SM2, and colistin and SM3, respectively. Similarly, the expression level of *pmrH* was 16.2 ± 25.0 -, 5.8 ± 2.5 -, and 2.1 ± 0.2 -fold higher in APEC isolates

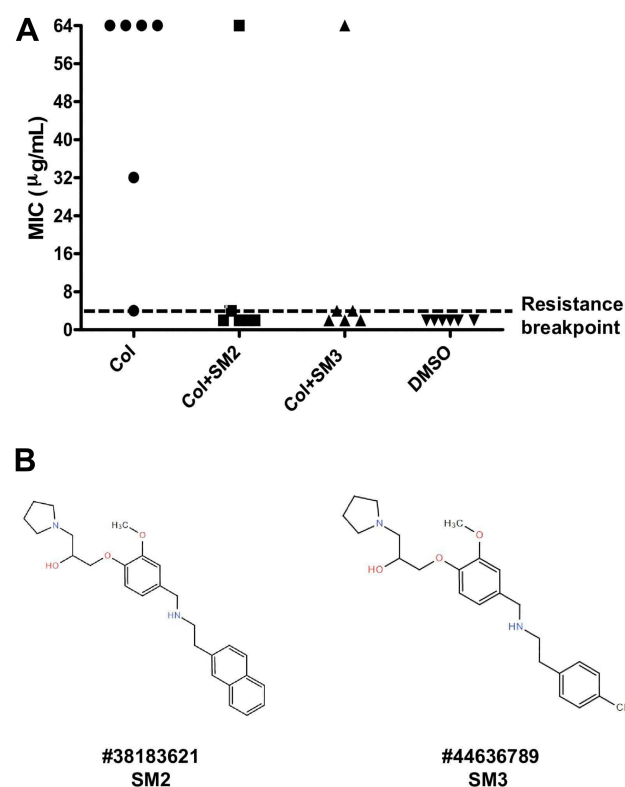


Figure 2 (A) Minimum inhibitory concentration (MIC) of APEC cultures passaged only in presence of colistin or in combination with SMs. Six parallel independent APEC cultures (Col 1–6, Col + SM2 1–6, and Col+ SM3 1–6) were serially passaged (8 passages) in M63 media starting with the sub-inhibitory concentration of colistin (0.0625 µg/mL) to up to 8 µg/mL (beyond resistance breakpoint) in presence and absence of 0.5× MIC of SMs. APEC cultures containing no colistin and no SM (1% DMSO; DMSO 1–6) were used as controls and passaged similarly to cultures containing colistin or colistin and SMs. The black dotted line indicates the MIC breakpoint for colistin resistance. **(B)** Chemical structure and ChemBridge ID of selected SMs.

passaged with colistin, colistin and SM2, and colistin and SM3, respectively. A similar trend was observed in *pmrA* and *pmrB* expression levels. The 3.6 ± 0.7 -, 1.9 ± 0.1 -, and 2.5 ± 0.1 -fold higher *pmrB* expression was observed in APEC isolates passaged with colistin, colistin and SM2, and colistin and SM3, respectively, as compared to control isolates, whereas 1.4 ± 0.2 -, 0.8 ± 0.3 -, and 1.3 ± 0.2 -fold higher *pmrA* expression was observed in APEC isolates passaged

with colistin, colistin and SM2, and colistin and SM3, respectively. Even though slight upregulation in the expression of *pmrCAB* and *pmrH* genes was observed in APEC isolates passaged with colistin in combination with SMs as compared to control isolates, the expression was significantly lower ($P < 0.05$) as compared to isolates passaged with colistin alone.

Mutations in Hypothetical and Several Phage-Related Proteins Were Identified in Col^R APEC Isolates Concurrent with *pmrAB* Mutations

In addition to *pmrB* and *pmrA*, non-synonymous and synonymous mutations were also observed concurrently in Col^R isolates in other genes encoding hypothetical proteins, several phage-related proteins [putative transposases, phage replication protein O, antitermination protein Q from phage origin, Rz endopeptidase from lambdoid prophage DLP12, lambdoid prophage DLP12 Bor-like protein, bacteriophage lysis protein, phage repressor protein, terminase large subunit, restriction alleviation protein, increased serum survival (Iss), and putative portal protein], uroporphyrinogen III C-methyltransferase, YD repeat protein, and electron transport complex protein RnfC (Figure 3, Table 5, Table S7, Dataset 1, Dataset 2). The details (protein id, type and region of mutations, amino acid changes) of the non-synonymous mutations observed in Col^R isolates compared to the Col^S isolates, including control isolates, are tabulated in Table 5 and Dataset 1. SNVs, MNV (multi-nucleotide variation), deletion, and insertion were observed in genes encoding hypothetical proteins (AGC85542.1, AGC85972.1, AGC85973.1, AGC85984.1). Interestingly, multiple mutations were detected in Col^R isolates in genes encoding several phage-related proteins. SNVs and MNVs were observed in genes encoding putative transposases (AGC87419.1). SNV and insertion were observed in genes encoding phage replication protein O (AGC84960.1). Deletion was observed in genes encoding bacteriophage lysis

Table 4 Mutations Observed in Col^R APEC Isolates Analyzed Using PointFinder Database

Isolate	Mutation	Nucleotide Change	Amino Acid Change
Col-1	<i>pmrA</i> p.A80V	GCT -> GTT	A -> V
Col-3	<i>pmrB</i> p.T92P	ACC -> CCC	T -> P
Col-5	<i>pmrB</i> p.L14Q	CTG -> CAG	L -> Q
Col-6	<i>pmrB</i> p.L14Q	CTG -> CAG	L -> Q
Col+SM2-2	<i>pmrB</i> p.L14Q	CTG -> CAG	L -> Q
Col+SM3-5	<i>pmrB</i> p.L14Q	CTG -> CAG	L -> Q

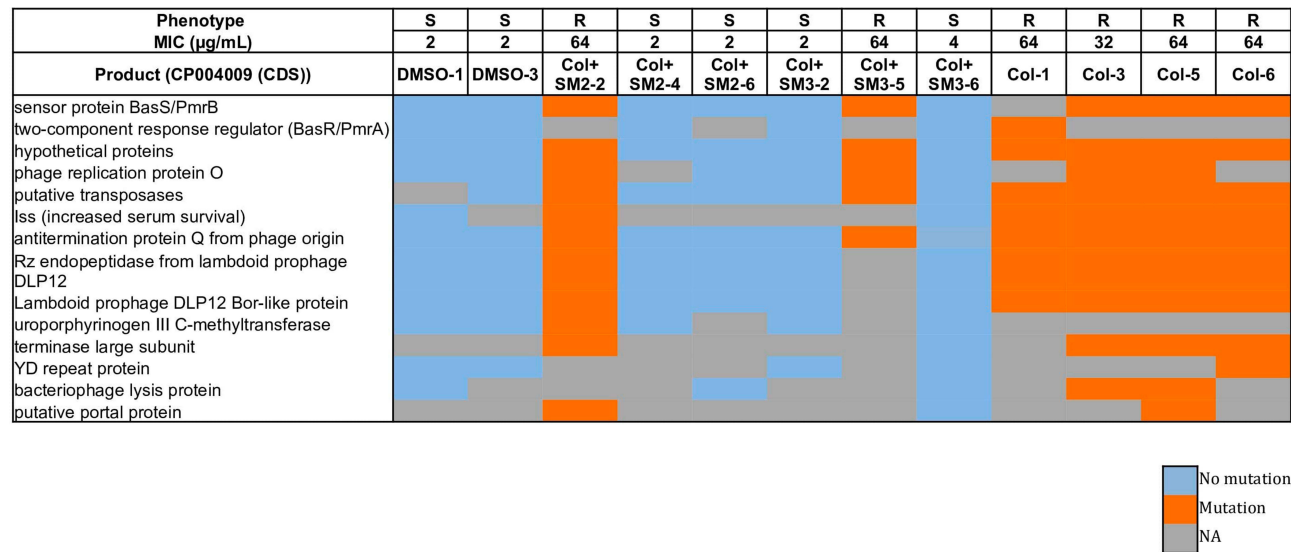


Figure 3 Heatmap displaying the non-synonymous mutational comparison between Col^R isolates (Col-1, Col-3, Col-5, Col-6, Col + SM2-2 and Col + SM3-5) as compared to Col^S (DMSO-1, DMSO-3, Col + SM2-4, Col + SM2-6, Col + SM3-2 and Col + SM3-6) isolates. Phenotype “R” indicates resistant APEC isolate and “S” indicates susceptible APEC isolate.

Abbreviation: NA, not available.

protein (AGC85980.1) and Iss (AGC85979.1). Likewise, SNVs and MNVs were observed in genes encoding antitermination protein Q from phage origin (AGC86222.1). Similarly, SNVs, deletion, and insertion were observed in genes encoding Rz endopeptidase from lambdoid prophage DLP12 (AGC86226.1), whereas SNV, MNV, and deletion were observed in genes encoding lambdoid prophage DLP12 Bor-like protein (AGC86227.1). Further, SNVs, insertion, and replacement were observed in genes encoding terminase large subunit (AGC87037.1). SNV was also observed in the gene encoding the putative portal protein (AGC87035.1). Similarly, insertion was observed in genes encoding uroporphyrinogen III C-methyltransferase (AGC89286.1), whereas SNV was observed in genes encoding YD repeat protein (AGC86138.1). Similar to non-synonymous mutations, synonymous mutations (only SNVs) were observed in hypothetical proteins, several phage-related proteins (putative transposases, phage replication protein O, antitermination protein Q from phage origin, Rz endopeptidase from lambdoid prophage DLP12, lambdoid prophage DLP12 Bor-like protein, bacteriophage lysis protein, phage repressor protein, terminase large subunit, restriction alleviation protein, and Iss), and electron transport complex protein RnfC (Table S7, Dataset 2). The role of these mutations in colistin-resistant bacteria has not been described before; however, these mutations being simultaneously present with *pmrAB* system could be adaptive mutations induced to compensate colistin stress or secondary to *pmrAB* mutations and induced lipid

A modifications. Further investigation will aid in an in-depth understanding of molecular sequelae associated with colistin resistance.

Discussion

Colistin is a last-resort antibiotic to treat diverse Gram-negative bacterial pathogens in humans and food animals.^{1,3} However, chromosomal- and plasmid-mediated resistance to colistin has been reported worldwide, which can limit the future use of colistin.^{2,4,11} The discovery of colistin activity enhancers or colistin adjuvants as a combination therapy is therefore crucial to extend the lifespan of colistin.⁴⁹ Strikingly, the highest prevalence of colistin resistance is reported in pathogenic *E. coli* strains (54%) isolated from food animals,¹¹ which necessitates the development of strategies to attenuate colistin resistance in pathogenic *E. coli* strains in food animals. Our study has identified SMs (SM2 and SM3) with novel pyrrolidinyl scaffold that potentiated the efficacy of colistin against APEC in vitro and in a wax moth larva model (Table 1, Figure 1). Our previous study had identified that these SMs affect the APEC membrane (by forming membrane blebs)²³ which might be the reason behind potentiating the activity of colistin, which is also a membrane-acting antibiotic.² These SMs also potentiated the activity of tetracycline and ciprofloxacin in vitro which might be due to increased intracellular uptake of tetracycline and ciprofloxacin due to breach in the membrane permeability caused by the SMs.^{23,50} Similar to our findings, multiple

Table 5 Non-Synonymous Mutations Observed in Col^R APEC Isolates

Product (Protein Id)	Type of Mutations	Amino Acid Changes
Sensor protein BasS/PmrB [AGC85036.1]	SNVs (A to T, T to G)	L14Q, T92P
Two-component response regulator BasR/PmrA [AGC85037.1]	SNV (G to A)	A80V
Hypothetical proteins [AGC85973.1, AGC85984.1, AGC85542.1, AGC85972.1]	SNVs, MNV, deletions, insertion	R15L, Q92R, D27N, A496_Q527del, A55_D59dup, S40fs
Putative transposases [AGC87419.1]	SNVs, MNVs	C22R, G33P, H219Y, E179D, V177I, N76K, R18L, K201R, L190T, A157V
Phage replication protein O [AGC84960.1]	SNVs, insertion	H152Q, S101A, A55_D59dup
Antitermination protein Q from phage origin [AGC86222.1]	SNVs, MNVs	D9E, A15V, I20V, V21G, S27A, G30R, C33G, C33_C34delinsWR, L41_R42delinsRL, I49V, I50V, G37C, Y38_I39delinsWV, A56M, E51D
Increased serum survival (Iss) [AGC85979.1]	deletion	*98fs
Rz endopeptidase from lambdoid prophage DLP12 [AGC86226.1]	SNVs, deletion, insertion	*154fs, E142*, T144A, R125Q, T129fs, T129S
Lambdoid prophage DLP12 Bor-like protein [AGC86227.1]	SNV, MNV, deletion	*98fs, L83F, K49E
Bacteriophage lysis protein [AGC85980.1]	Deletion	*154fs
Putative portal protein [AGC87035.1]	SNV	R237S
Terminase large subunit [AGC87037.1]	SNV, insertion, replacement	W318fs, M308V
YD repeat protein [AGC86138.1]	SNV	A68G
Uroporphyrinogen III C-methyltransferase [AGC89286.1]	Insertion	P389_Q390insAPAP

Note: *Stop codon.

Abbreviations: del, deletion; ins, insertion; dup, duplication; fs, frame shift.

membrane-active compounds with synergistic interaction with colistin have been identified (Table 6) in previous studies.^{13,15,19,24,25,27} A urea-containing class of 2-aminoimidazole-based adjuvants potentiated colistin activity against Col^S *A. baumannii* with a reduction of colistin MIC by 1000-fold, and increased survival (36–50%) of *G. mellonella* larva by exhibiting a membrane-permeabilizing effect in the bacteria.¹³ Eugenol, an essential oil derived from clove, also potentiated the activity of colistin (MIC reduction up to 8-fold) against multiple Col^R *E. coli* isolated from different animals (duck, goose, avian, porcine, zebra, fox, and peacock) by affecting the bacterial membrane integrity.²¹ Similarly, a combination of pentamidine, a bacterial outer-membrane-active compound, was effective to treat Col^R *A. baumannii* infection in mice.¹⁹ Protegrin-1, an

antimicrobial peptide derived from porcine leucocytes which disrupts the bacterial membranes, also synergized with colistin against both Col^S and Col^R *A. baumannii* strains.²⁵ In the same way, cationic antimicrobial peptides (CAMPs) such as LL-37 (human cathelicidin) and CAMA (cecropin) also reduced the MIC of colistin by at least eight-fold against methicillin-resistant *Staphylococcus aureus* and MDR *P. aeruginosa*.²⁶ Further, daptomycin, a membrane-acting antibiotic, also showed synergistic interaction with colistin against MDR *A. baumannii*.²⁴ Moreover, the screening of commercially available FDA-approved library (Prestwick chemical library) identified five compounds (clomiphene citrate, mitoxantrone dihydrochloride, methyl benzethonium chloride, benzethonium chloride, and auranofin) that potentiated the activity of colistin against *P. aeruginosa*.²⁷

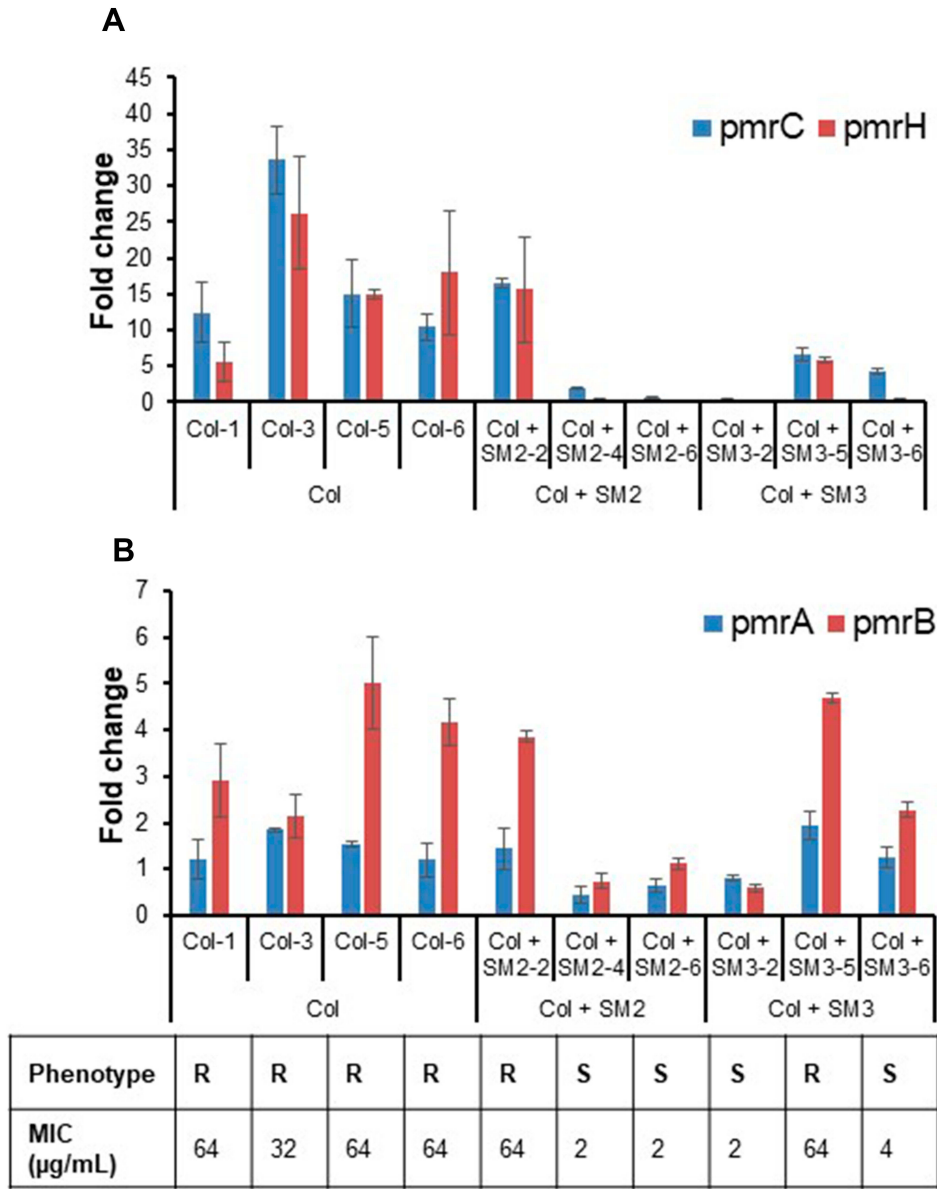


Figure 4 (A) Expression (fold change) of *pmrC* and *pmrH* in APEC isolates passed with colistin alone (Col-1, Col-3, Col-5 and Col-6), colistin and SM2 (Col + SM2-2, Col + SM2-4 and Col + SM2-6), and colistin and SM3 (Col + SM3-2, Col + SM3-5 and Col + SM3-6) as compared to control (DMSO-1 and DMSO-3) isolates. **(B)** Expression (fold change) of *pmrA* and *pmrB* in APEC isolates passed with colistin alone (Col-1, Col-3, Col-5 and Col-6), colistin and SM2 (Col + SM2-2, Col + SM2-4 and Col + SM2-6), and colistin and SM3 (Col + SM3-2, Col + SM3-5 and Col + SM3-6) as compared to control (DMSO-1 and DMSO-3) isolates. Phenotype “R” indicates resistant APEC isolate and “S” indicates susceptible APEC isolate.

Similarly, four compounds (compounds 1, 2, 3, and 4) identified through screening of substituted 2-aminoimidazole analogs and nitrogen-dense heterocycle libraries reduced the MIC of colistin against Col^S and Col^R *K. pneumoniae*, *A. baumannii*, and *E. coli* by >32-fold at 30 µM concentrations.²² Niclosamide, an anthelmintic drug, also synergized with colistin against Col^R *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *E. coli*, and *Enterobacter cloacae* with reduction of colistin MIC by 64-fold.¹⁸ Resveratrol, a compound derived from plant, synergized with colistin against Col^S and Col^R *K. pneumoniae*,

A. baumannii, *E. coli*, *E. cloacae*, *Citrobacter braakii*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Proteus mirabilis*.¹⁷ In addition, N-acetylcysteine, a mucolytic agent, also synergized with colistin against Col^S and Col^R *A. baumannii* grown in planktonic phase and in biofilms.¹⁶ These findings suggest that the combination of colistin with other membrane-active compounds or drugs can result in synergistic interaction by facilitating the colistin effect on bacterial membranes.⁵¹ In this study, we only tested the synergistic potential of SMs against three APEC strains (O78, O1, and O2); however, these

Table 6 Summary of Colistin Adjuvants Tested Against Different Pathogenic Bacteria

Bacteria	Adjuvant Compound	Increase in Efficacy in vitro	Increase in Efficacy in vivo	Reference
Col ^S <i>A. baumannii</i>	Urea containing class of 2-aminoimidazoles	MIC reduction by 1000-fold	Increased survival (36–50%) of <i>G. mellonella</i> larva	[13]
Col ^R <i>E. coli</i>	Eugenol	MIC reduction up to 8-fold	NA	[21]
Col ^R <i>A. baumannii</i>	Pentamidine	NA	Rescued (10 of 11) mice infected with Col ^R <i>A. baumannii</i>	[19]
Col ^S and Col ^R <i>A. baumannii</i>	Protegrin-I	Synergistic (FIC<0.5)	NA	[25]
Methicillin-resistant <i>S. aureus</i> and MDR <i>P. aeruginosa</i>	LL-37, cecropin	MIC reduction by at least eight-fold	NA	[26]
MDR <i>A. baumannii</i>	Daptomycin	Synergistic (≥2 log reduction compared to colistin alone)	NA	[24]
<i>P. aeruginosa</i>	Clomiphene citrate, mitoxantrone dihydrochloride, methyl benzethonium chloride, benzethonium chloride, and auranofin	Synergistic (FIC<0.5)	NA	[27]
Col ^S and Col ^R <i>K. pneumoniae</i> , <i>A. baumannii</i>	Compound 1, 2, 3 and 4	MIC reduction by >32-fold	NA	[22]
Col ^R <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>A. baumannii</i> , <i>E. coli</i> , and <i>Enterobacter cloacae</i>	Niclosamide	MIC reduction by 64-fold	NA	[18]
Col ^S and Col ^R <i>K. pneumoniae</i> , <i>A. baumannii</i> , <i>E. coli</i> , <i>E. cloacae</i> , <i>Citrobacter braakii</i> , <i>Stenotrophomonas maltophilia</i> , <i>Serratia marcescens</i> , and <i>Proteus mirabilis</i>	Resveratrol	Synergistic (FIC<0.5)	NA	[17]
Col ^S and Col ^R <i>A. baumannii</i>	N-acetylcysteine	Synergistic (FIC<0.5)	NA	[16]
Col ^S and potentially Col ^R <i>E. coli</i>	Pyrrolidiny SMs (SM2 and SM3)	MIC reduction by at least 10-fold	Twofold (>4.5 logs reduction compared to colistin alone), increased survival (>45%) of <i>G. mellonella</i> larva	This study

Abbreviation: NA, not available.

SMs were previously found to be effective against multiple APEC strains, including antibiotic-resistant and biofilm protected APEC;²³ therefore, we believe that these SMs may potentiate the activity of colistin against diverse *E. coli* and possibly against other Gram-negative pathogens.

However, this needs further investigation. Further, SMs other than SM2 and SM3 (SM4, SM5, SM6, SM7, SM9, and SM11) which have synergistic interaction with colistin, can be also investigated for their potential as colistin adjuvants.

This study identified mutations in *pmrB* and *pmrA* in Col^R *E. coli* O78 (Figure 3, Tables 4 and 5). In this study, substitutions in *pmrB* p. L14Q, *pmrB* p. T92P, and *pmrA* p. A80V were observed. These substitutions are predicted deleterious (PROVEAN score; L14Q: −4.222, T92P: −3.783, and A80V: −3.818; <http://provean.jcvi.org>)⁵² to the functions of PmrB and PmrA proteins. In other studies, different *pmrB* and *pmrA* mutations (Table 7) responsible for colistin resistance in *E. coli* were observed.^{48,53,58} In Col^R *E. coli* O25 isolated from human clinical specimens in Japan, deletion (Δ27–45, LISVFWLWHESTEQIQLFE) in *pmrB* and substitution (L105P) in *pmrA* were reported.⁵³ In

Table 7 Summary of *pmrB* and *pmrA* Mutations Reported in Col^R *E. coli*

Bacteria/ Strain/Source	Observed Mutations	Reference
<i>E. coli</i> O25 (human clinical specimens)	Deletion Δ27–45 LISVFWLWHESTEQIQLFE; <i>pmrB</i>	[53]
<i>E. coli</i> O25 and O18 (human clinical specimens)	G206D; <i>pmrB</i>	[53]
<i>E. coli</i> O25 (human clinical specimens)	L105P; <i>pmrA</i>	[53]
<i>E. coli</i> LC711/14 (urinary tract infected human patient)	L10P; <i>pmrB</i>	[48]
<i>E. coli</i> (human clinical specimens)	A118T, E123D, Y315F, and Y358N; <i>pmrB</i>	[54]
<i>E. coli</i> (Parisian fecal samples)	L10P, L10R, C84R, C84Y, P94A, P94L, P94Q, E121K, E121Q, A159P, and A159V; <i>pmrB</i> G53A, G53C, G53E, G53R, G53S, G53V, G53W, R81L, and R81S; <i>pmrA</i>	[57]
<i>E. coli</i> (swine)	S138N, E123D, T156A, G160E, V161G, and V351I; <i>pmrB</i> G144S, S39I, and R81S; <i>pmrA</i>	[55,56]
<i>E. coli</i> (calf cecum samples)	D283G and Y358N; <i>pmrB</i>	[58]
<i>E. coli</i> (poultry farms)	P94L and D283G; <i>pmrB</i> S29G; <i>pmrA</i>	[59]

the same study, substitution (G206D) was observed in *pmrB* in Col^R *E. coli* O25 and O18 isolates.⁵³ These mutations have led to modification of lipid A with the addition of PETN and upregulation of *eptA* (*pmrC*) and *arnT* expression and thereby resistance to colistin.⁵³ Likewise, in Col^R *E. coli* LC711/14 isolated from a patient with a urinary tract infection in Italy, substitution (L10P) in *pmrB* was reported, this mutation upregulated the expression of *pmrHFIJKLM* operon resulting in lipid A modification and colistin resistance.⁴⁸ Similarly, in Col^R *E. coli* isolated from human clinical specimens in China, multiple substitutions (A118T, E123D, Y315F, and Y358N) in *pmrB* were observed, these mutations were predicted to affect phosphate transfer in *pmrAB* thus altering the function of PmrB protein.⁵⁴ Likewise, in Col^R *E. coli* isolated from Parisian fecal samples, different substitutions in *pmrB* (L10P, L10R, C84R, C84Y, P94A, P94L, P94Q, E121K, E121Q, A159P, and A159V) and *pmrA* (G53A, G53C, G53E, G53R, G53S, G53V, G53W, R81L, and R81S) were observed, these mutations were also predicted to impact the functions of PmrB and PmrA proteins.⁵⁷ Interestingly, different substitutions in *pmrB* and *pmrA* have been observed in Col^R *E. coli* isolated from different food animals.^{55,58} In Col^R *E. coli* isolated from swine, different *pmrB* (S138N, E123D, T156A, G160E, V161G, and V351I) and *pmrA* (G144S, S39I, and R81S) substitutions were observed.^{55,56} Whereas in Col^R *E. coli* isolated from calf cecum samples, only substitutions in *pmrB* (D283G and Y358N) were observed.⁵⁸ Further, in a recent study in Senegalese poultry farms, Col^R *E. coli* isolated from healthy chickens having substitution in *pmrB* (P94L and D283G) and *pmrA* (S29G) has been reported.⁵⁹ However, *pmrB* and *pmrA* mutations observed in this study have not been reported yet in any other studies. Based on the studies described above, it is suggested that the different *pmrB* and *pmrA* mutations might be responsible for colistin resistance in different *E. coli* isolates, which might depend upon the *E. coli* serotype/strain, source of isolation and other factors, including the type of treatment regimen followed in that location. The findings of this study can therefore provide insights for further investigation of potential mechanisms of chromosomal-mediated colistin resistance in poultry *E. coli* isolates.

The SMs identified in this study decreased the frequency of colistin resistance evolution (Figure 2). SM combination also downregulated the *pmrCAB* and *pmrH* expression (Figure 4); therefore, these SMs can function as anti-evolution drugs that slow colistin resistance development.³² The upregulation of the expression

of operons *pmrCAB* and *pmrHFIJKLM* is crucial for bacteria to acquire colistin resistance; therefore, drugs targeting *pmrCAB* and *pmrH* expression might help to overcome colistin resistance.^{45,48} The induced *pmrCAB* and *pmrH* expression specifically increases the ability of bacteria to survive in the presence of colistin.^{45,48} Similar to our SMs, a combination of membrane-acting 2-aminoimidazole-based compounds with colistin also resulted in no resistant *A. baumannii* and *K. pneumoniae*, which is mediated by downregulation of *pmrCAB* expression.²⁰ The lack of resistance observed might be due to reduced selection pressure in combination treatment compared to colistin treatment alone.²⁰ Similarly, antimicrobial peptide glycine-leucine-amide (PGLA), which affects bacterial outer membrane, combination with ciprofloxacin and tobramycin resulted in a 30-fold decrease in *E. coli* resistance compared to ciprofloxacin and tobramycin alone treatment.⁴¹ The combination treatment might reduce the number of resistance-conferring mutations, which ultimately reduces the resistant bacterial population, which is also likely to have occurred in our study.⁴¹ Compounds, 1, 2, and 3 (substituted 2-aminoimidazole analogs and nitrogen-dense heterocycles), also suppress the colistin resistance by abolishing or reducing the extent of lipid A modification induced by *pmrAB* system and *mcr-1* gene.²² These findings suggested that the combination of membrane-active adjuvants with conventional antibiotics, including colistin, is a practical way to attenuate colistin and antibiotic resistance as a whole.

Besides *PmrA* and *PmrB*, this study also identified other mutations in different hypothetical and phage-related proteins in Col^R isolates (Figure 3, Table 5). Similar to our findings, mutations in hypothetical proteins were also found in Col^R isolates of *A. baumannii*,⁶⁰ *K. pneumoniae*,⁶¹ and *P. aeruginosa*.⁶² In our study, we did not obtain any isolates possessing mutations only in genes other than the *pmrAB* system to determine the relevance of these hypothetical and phage proteins in colistin resistance; therefore, the significance of these mutations is unknown at this time. It is likely that these mutations might just represent adaptive mutations induced to compensate colistin stress or secondary to *pmrAB* mutations and induced lipid A modifications. The induction of prophage to adapt antibiotic stress was observed when *P. aeruginosa* was exposed to colistin,⁶³ which can support our finding. Further investigation is necessary to understand the precise role of these mutations in colistin

resistance which can help in understanding molecular sequelae associated with colistin resistance.

Conclusion

In conclusion, our study revealed potential mechanisms for chromosomal-mediated colistin resistance in APEC. Two SMs (SM2 and SM3) containing pyrrolidinyl scaffold were identified that potentiated the colistin activity, attenuated the colistin-resistance development against APEC, and downregulated the *pmrCAB* and *pmrH* expression. Previously unknown mutations potentially responsible for colistin resistance in *pmrB* and *pmrA* were identified in Col^R APEC isolates. Mutations were also identified in hypothetical and phage-related proteins; however, further studies are needed to elucidate the relevance of these mutations in colistin resistance. In addition, testing colistin adjuvant properties of these SMs against other APEC related pathogens, including human ExPECs and other Gram-negative pathogens, will have a wider impact on prudent use of colistin.

Data Sharing Statement

All relevant data are contained within the manuscript.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, agree to the journal in which paper was submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest.

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