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ORIGINAL ARTICLE

Activity of levofloxacin in combination with colistin against *Acinetobacter baumannii*: *In vitro* and in a *Galleria mellonella* model

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Abstract *Background/Purpose:* Treatment of *Acinetobacter baumannii* infections is challenging owing to widespread multidrug-resistant *A. baumannii* (MDR-AB) and the lack of novel agents. Although recent data suggest that levofloxacin (LVX) may have unique activity against MDR-AB in combination with colistin (CST), further preclinical work is needed.
Methods: We used a *A. baumannii* type strain ATCC19606, a CST-resistant strain AB19606R, and two clinical isolates (GN0624 and GN1115) of MDR-AB to investigate the *in vitro* and *in vivo* efficacy of LVX–CST combination. Synergy studies were performed using the microtiter plate chequerboard assay and time–kill methodology. Inhibitory activity of antibiotics against biofilms and the mutant prevention concentrations were also studied *in vitro*. A simple invertebrate model (*Galleria mellonella*) has been used to assess the *in vivo* activity of antimicrobial therapies.
Results: The LVX–CST combination was bactericidal against the CST-susceptible clinical isolate (GN0624). In checkerboard assays, synergy (defined as a fractional inhibitory concentration index of < 0.5) was observed between CST and LVX in GN0624. The combination had antibiofilm properties on the preformed biofilms of four tested strains and could prevent the emergence of CST-resistant *A. baumanni*. Treatment of *G. mellonella* larvae infected with lethal doses of *A. baumannii* resulted in significantly enhanced survival rates when LVX was given with CST compared with CST treatment alone ($p < 0.05$).

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Conclusion: In summary, a synergistic or additive effect between CST and LVX was observed *in vitro* and *in vivo* against CST-susceptible *A. baumannii* strains, although not against CST-resistant ones.

Introduction

During the past few decades, *Acinetobacter baumannii* has become a pathogen of increased clinical importance due to its remarkable ability to cause outbreaks of infections and to acquire resistance to almost all currently used antibiotics, including the carbapenems. The emergence of multidrug-resistant *A. baumannii* (MDR-AB) further limits the treatment options and constitutes a serious threat to international public health.[1] The only agents that remain consistently active *in vitro* against MDR-AB are the polymyxins and tigecycline. However, resistance to colistin (CST) and tigecycline have been increasingly reported.[2,3] With very few options now left, unorthodox combination therapies that include CST for the treatment of infections caused by MDR-AB are increasingly being considered, although comprehensive clinical data are lacking.[4]

Levofloxacin (LVX) is one of the outstanding representatives of the third generation of quinolone antibiotics that have been a useful class of broad-spectrum antimicrobials. But declining susceptibilities may have implications for the empiric use of fluoroquinolones in patients at risk of developing infections often caused by *A. baumannii*.[5] However, the presented study indicated that the LVX—CST combination was shown to have activity against CST-susceptible (CSTs), MDR clinical strains, in accordance with a previous study.[6] Although this combination appears to be a promising treatment option, further preclinical work is clearly needed before it can be considered for clinical use.

Animal studies investigating new or unconventional antimicrobial therapies are necessary in order to inform on appropriate dosage, potential toxicity, and *in vivo* efficacy. However, mammalian models of infection are associated with high cost, ethical constraints, and specialized training requirements.[7] Therefore, alternative infection models using insects are being increasingly employed to characterize virulence of bacterial pathogens and to evaluate novel therapeutics prior to characterization in mammalian models. The introduction of a *Galleria mellonella* model of *A. baumannii* infection may prove useful to this end. Like other nonmammalian infection models, microbial virulence is similar in *G. mellonella* and mammals, and this model has already been used to determine the virulence of various human pathogens including *A. baumannii*.[8] In this study, we aimed to evaluate *G. mellonella* as a model to study the *in vivo* efficacy of LVX—CST combination.

Methods

Antibiotics

CST and LVX were commercially obtained from Sigma-Aldrich (Shanghai, China). Stock solutions were prepared according to the Clinical and Laboratory Standards Institute (CLSI) guidelines in the appropriate solvent, following which the solution was stored at 4°C for up to 1 month, conditions under which the drugs were stable.

Bacterial isolates and susceptibility testing

Isolates of *A. baumannii* used in this study were two MDR-clinical isolates, CSTs strain GN0624 and CST-resistant (CSTr) strain GN1115, as well as the *A. baumannii* type strains ATCC 19606 and AB19606R as controls. GN0624 was isolated from the urine of a hospitalized patient who was treated in the intensive care unit. GN1115 was isolated from the sputum of a neurosurgical patient. Both of the two patients were hospitalized in a tertiary-care hospital located in Anhui, China, in 2013. GN0624 and GN1115 were identified by API 20NE (BioMérieux, Marcy-l'Étoile, France) and species-specific polymerase chain reaction for the *blaOXA-51-like* gene.[9] Minimum inhibitory concentrations (MICs) of CST and LVX were obtained by the standard agar dilution method according to CLSI recommendations. Interpretation criteria for susceptibility tests were based on CLSI guidelines.[10] Genes encoding resistance to carbapenems (*blaOXA-23-like*, *blaOXA-24-like*, *blaOXA-51-like*, *blaOXA-58-like*, and *metallo-β-lactamase* genes) were identified with polymerase chain reaction and sequencing as previously described.[11]

LVX—CST checkerboard assays

Synergy between two agents of CST and LVX was assessed for a range of concentrations (between 0 and 8 × MICs) using the checkerboard method. In brief, 96-well microtiter plates (Sigma-Aldrich) were set up with increasing concentrations of CST (0—512 mg/L) in each column and LVX (0—1024 mg/L) in each row. Wells were inoculated with 5×10^5 CFU/mL of the test organism and incubated at 37°C for 18 hours.[12] The fractional inhibitory concentration index (FICI) and the susceptibility breakpoint index (SBPI) were calculated. The FICI was interpreted as follows: synergy, FICI $\leq$ 0.5; antagonism, FICI $>$ 4.0; and indifferent,

$0.5 < FICI < 4.0$. Synergy by checkerboard assay was also defined as a SBPI of > 2.[7,13]

Time–kill assays

Time–kill assays were conducted for each strain using CST alone and in combination with LVX according to a previously described methodology.[14] Drug concentrations of CST and LVX in the following studies were adjusted to the susceptible breakpoint concentration of the two drugs recommended by the CLSI, i.e., CST 2mg/L, LVX 2mg/L. Tubes containing Mueller–Hinton broth (MHB; Oxoid Ltd., Cambridge, UK) with and without (growth control) antibiotic were seeded with a logphase inoculum of roughly 5×10^5 CFU/mL to a final volume of 10 mL. Inoculated broths were incubated in an ambient atmosphere at 37°C. Bacterial counts were measured at selected time intervals of 0 hours, 4 hours, 8 hours, 24 hours by enumerating the colonies in 10-fold serially diluted specimens of 100 μL aliquots plated on Mueller–Hinton agar (MHA; Oxoid Ltd.) at 37°C.[15] Synergism between two antimicrobials as any ≥ 2 $\log_{10}$ decrease of bacterial growth compared with the most active single agent and the number of surviving organisms in the presence of the combination must be ≥ 2 $\log_{10}$ CFU/mL below the starting inoculum.[12] Bactericidal activities of drug combinations were defined as ≥ 3 $\log_{10}$ CFU/mL reduction compared with the most active drug at 24 hours.[16] All experiments were performed in duplicate.

Biofilm formation and quantification

For this study, four *A. baumannii* stains mentioned above were cultured in MHB supplemented with 0.2% glucose. *Pseudomonas aeruginosa* NCTC-27853 were used as a positive control. Bacteria were enriched for 1 day at 37°C in 5 mL MHB and adjusted to 0.5 McFarland standards. Cultures were then diluted 1:100 in MHB supplemented with 0.2% glucose (1×10^6 CFU/mL) and 200 μL was added to each well of a 96-well tissue culture-treated polystyrene plate. Negative controls contained only MHB. After incubation at 37°C for 24 hours, the culture medium was removed from each well and plates were washed twice with phosphate-buffered saline (PBS) to remove free-floating planktonic cells. The biofilms formed on the well bottoms were then fixed by 100 μL methanol (100%) and incubation for 15 minutes. After removing the methanol, the biofilm in each plate was stained with 0.4% crystal violet (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) solution (w/v) for 30 minutes. The excess stain was then thoroughly rinsed away with distilled water and the plates were air dried. Once dry, 33% (v/v) glacial acetic acid was added to each well. The average optical density values were calculated for all tested strains and negative controls. These optical density were measured at 595 nm (OD595 values) and were considered to be a measure of the bacteria adhering to the surface and forming biofilms. To compensate for background absorbance, OD595 readings from sterile medium, fixative, and dye were averaged and were subtracted from all test values.[17,18]

Assay of the susceptibility of biofilm forming strains to antibiotics

Biofilms of individual strains were prepared in 96-well tissue culture-treated polystyrene plate. The medium in the well was removed with aspiration and the biofilm was treated either with CST or LVX alone or in combination. CST and LVX alone (2 mg/L, 200 μL) or their combination (2×2 mg/L, 200 μL) were added to bacterial cells suspended in MHB supplemented with 0.2% glucose (1×10^6 CFU/mL). Wells in which no test agents were added served as controls. The medium was gently aspirated after 1 day at 37°C. Inhibition of biofilm growth was quantified using crystal violet staining as described above. A ratio of OD value (ODr) of biofilm formation for the isolate incubated with a drug to that without a drug (native biofilm) was calculated. Lower ODr values indicate greater inhibitory activity on biofilm-embedded *A. baumannii*. The percentage of biofilm inhibition was calculated using the following equation[19,20]:

$$[1 - (\text{OD595 of cells treated with test agent} / \text{OD595 of untreated control})] \times 100. \quad (1)$$

Experiments were performed in triplicate and the data were averaged. The results were expressed as a percentage of biofilm inhibition.

The mutant prevention concentrations

The MPCs of the two drugs alone and in combination were determined using a modified method described by Zhao and Drlica[21] and Zhanel et al.[22] In brief, two CSTs isolates (ATCC19606, GN0624) were grown overnight on MHA at 37°C in ambient air. The overnight growth was then swabbed into MHB and incubated for 3 hours at 37°C in ambient air in order to achieve inocula of $\sim 10^{10}$ CFU. Samples (100 mL) were inoculated onto MHA plates containing 0.5×, 1×, 2×, 4×, 8×, 16×, or 32× their CST MIC alone and in combination with LVX (2 mg/L). Finally, the inoculated plates were incubated for 48 hours at 37°C and screened visually for growth. Each reported data point is based on at least three independent experiments including the concentration steps above and below the MPC. The MPC was recorded as the lowest antimicrobial concentration at which no colonies grew on an agar plate after 48 hours of incubation. The MIC and MPC data were used to calculate the size of the mutant prevention index (MPI = MPC/MIC) for each strain and drug combination.

G. mellonella model of *A. baumannii* infection

Efficacy of CST or LVX in *A. baumannii* infection was tested using the *G. mellonella* infection model which was initially reported by Peleg et al.[23] Batches of *G. mellonella* (Kaide Ruixin Co., Ltd., Tianjin, China) were stored at 4°C in the dark and were used within 10 days of receipt. Larvae were selected to be 15–25 mm in length, having a cream color with minimal speckling and no grey markings. Caterpillar masses varied slightly but were typically 250 mg and this value was used to calculate treatment doses. To establish

the optimal inocula required for staggered killing of *G. mellonella* over 96 hours, eight caterpillars were inoculated with bacterial suspensions containing final concentrations of 10^4 CFU/larva, 10^5 CFU/larva, and 10^6 CFU/larva. A 50 μL Hamilton syringe (Hamilton, Shanghai, China) was used to inject 10-μL aliquots of the inoculum into the hemocoel of each caterpillar via the last left proleg. Caterpillars were incubated at 37°C and were observed daily for 96 hours. They were considered dead if they did not respond to touch.

Sixteen larvae of appropriate weight (0.25—0.35 g) were randomly selected to comprise each group. Larvae were inoculated with a lethal dose of the four strains described above followed by tested drug 2 hours after inoculation. Antibiotics were administered via 10 μL injections into the last right proleg. Drug doses were selected to be representative of those used to treat human infection and consisted of CST at 2.5 mg/kg, LVX at 6.7 mg/kg. Treatment was given only once. For all experiments, four control groups were used: the first group included caterpillars that were inoculated with PBS, the second group received the inoculum only, the third group received antimicrobial agent(s) only, and the fourth group included caterpillars that received no injection. If more than one larva from these groups died, the results were disregarded and the experiment was repeated. The caterpillars were observed for survival every 24 hours for 4 days. Experiments were performed three times on separate occasions.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Survival analysis was determined using the log-rank test, with a p value ≤ 0.05 considered statistically significant.

Results

Genotypic and phenotypic characteristics for the study strains

A. baumannii ATCC 19606 was susceptible to β-lactams, aminoglycosides, quinolones, polymyxins, and tigecycline. AB19606R was resistant to CST (MIC: $>$ 128 mg/L) but was susceptible to LVX (MIC $=$ 0.25 mg/L). GN0624 was resistant to all agents tested (including carbapenems) but was susceptible to CST (MIC $=$ 2 mg/L). GN1115 was resistant to all agents tested including CST (MIC $=$ 16 mg/L). *A. baumannii* GN0624 and GN1115 were positive for *blaOXA-51-like* and *blaOXA-23-like* genes, whilst *metallo-β-lactamase* genes were not detected (Table 1).

Chequerboard assays

In the chequerboard assays, no significant synergy were observed in ATCC19606 and AB19606R as assessed using the FICI (1.0625 and 0.5625) despite a 16-fold reduction in the MIC of CST in the presence of subinhibitory

Table 1 Summary of the synergy, time—kill, and biofilm inhibition assay results from combinations containing colistin and levofloxacin against four *Acinetobacter baumannii* strains.

A. baumannii isolate	Feature(s)	Susceptibility	MICs (μg/mL)			Biofilm inhibition (%)[b]			Synergy testing results		
			CST	LVX	CST + LVX	CST	LVX	CST + LVX	Lowest FICI[a]	SBPI[a]	Time—kill assay
ATCC 19606	Type strain	FEP, TZP, SCF, IPM, MEM, CIP, GEN, LVX, AMK, CST, TIG	0.5	0.25	0.03 + 0.25	—	—	20 ± 5.9	1.0625 (I)	74.7 (S)	Bactericidal, re-growth
Ab19606R	Screened out from ATCC 19606	LVX	128	0.25	8 + 0.125	—	14.5 ± 4.6	44.3 ± 8.7	0.5625 (I)	16.25 (S)	Inhibition of re-growth
GN0624	Clinical isolate bla_{OXA-23}, bla_{OXA-51}	CST	2	16	0.5 + 2	6.9 ± 1.7	10.3 ± 2.9	77.7 ± 10.5	0.37 (S)	5 (S)	Bactericidal, inhibition of re-growth
GN1115	Clinical isolate bla_{OXA-23}, bla_{OXA-51}	—	16	16	4 + 8	—	8.4 ± 2.3	61.9 ± 5.2	0.75 (I)	0.75	Growth

[a] As observed in three independent experiments.
[b] Mean from three replicate experiments. The biofilm inhibition of LVX—CST combination was higher than CST or LVX monotherapy ($p < 0.05$).
AMK = amikacin; CIP = ciprofloxacin; CST = colistin; FEP = cefepime; FICI = fractional inhibitory concentration index; GEN = gentamicin; IPM = imipenem; LVX = levofloxacin; MEM = meropenem; SBPI = susceptible breakpoint index; SCF = cefoperazone/sulbactam; TIG = tigecycline; TZP = piperacillin/tazobactam.

concentrations of LVX. The two clinical isolates (GN0624 and GN1115) had a four-fold decrease in CST MICs and had a reversal of LVX resistance with the change of LVX MICs from 16 mg/L to 2 mg/L and 8 mg/L, respectively. Synergistic activity in LVX–CST combination was observed in GN0624, which showed synergy in FICI (0.37), and in GN1115, which showed no interaction in FICI (0.75). However, a SBPI > 2 were observed in ATCC19606, AB19606R, and GN0624, indicative of a potent synergistic interaction (Table 1).

Time–kill assays

For the type strain ATCC19606, which was susceptible to most antibiotics, CST and LVX showed bactericidal activity with monotherapy. However, CST alone or in combination reduced the initial inoculums during the 1st 8 hours in this strain but the bactericidal effects were subsequently abolished after 24 hours of incubation and regrowth was detected. Both LVX alone and LVX–CST combination expressed bactericidal activity in the strain AB19606R, while CST alone showed no activity. A subinhibitory concentration of CST exhibited a rapid killing effect against GN0624, but this was not sustained, and the isolate was able to regrow at 24 hours to a bacterial density higher than the starting inoculum. When two agents were combined, however, the combination was consistently bactericidal and no regrowth was observed after 24 hours. There was a > 5 $\log_{10}$ CFU/mL difference in the viable counts compared with cells treated with CST only at 24 hours and a 3 $\log_{10}$ CFU/mL reduction compared with the starting inoculum. For the clinical isolate (GN1115), CST alone or in combination with LVX were unable to produce bacteriostatic or bactericidal activity during the present study time and regrowth was found at the 24th hour of incubation. Representative time–kill curves for four isolates of *A. baumannii* were shown in Figure 1. Logarithmic and fold changes of time–kill experiments at 24 hours were presented in Table 2.

Inhibitory activity of antibiotics against biofilms

All the tested strains showed biofilm production. In the biofilm model, susceptible breakpoint concentration of each antimicrobial agent was used to investigate the inhibitory effect. The use of CST as single drug treatment had a very slight effect in reducing the viable CFU of biofilm-embedded *A. baumannii* (GN0624). The ODr of GN0624 was 0.931 and the percentage of biofilm inhibition was 6.9. CST alone had no effect on the other three strains. Treatment with 2 mg/L of LVX alone had an inhibitory effect of 3–15% on preformed biofilms of the studied strains with the exception of ATCC19606 type strain. However, CST plus LVX exhibited enhanced antibacterial effects (ODr 0.8, 0.557, 0.223, and 0.381, respectively) compared with the single drug therapy. The ODr of studied strains with combined therapy were generally lower than those used as single agent (Table 1).

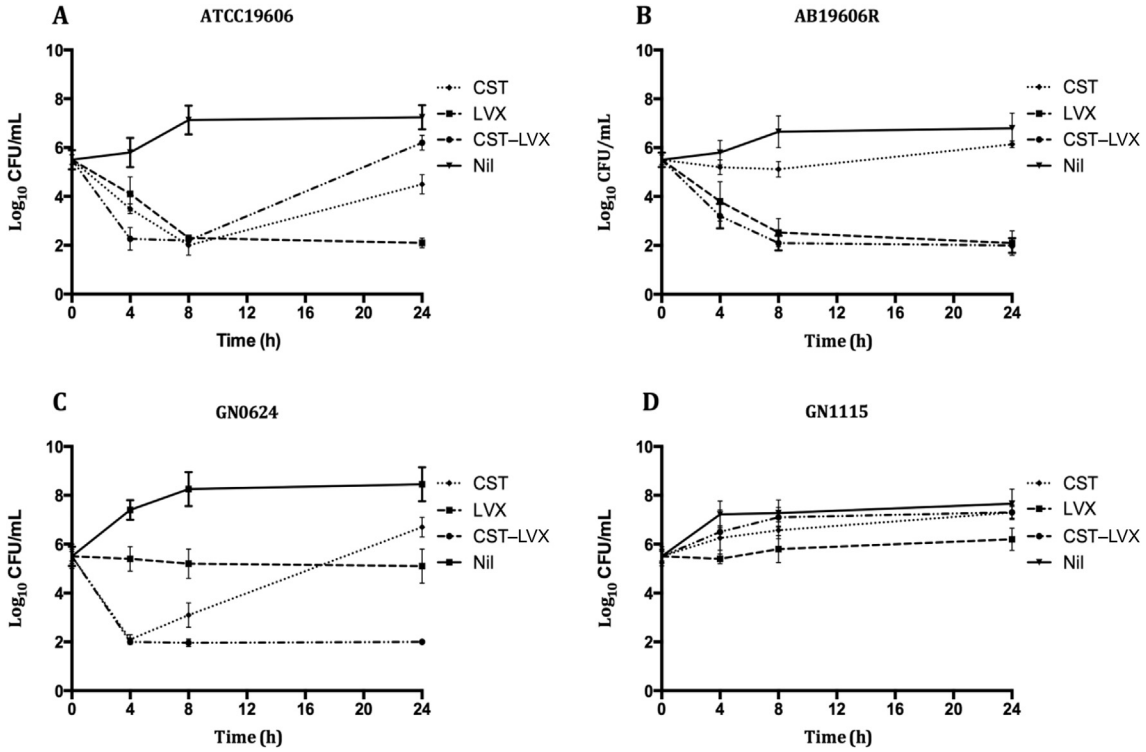

Figure 1. Time–kill assays for colistin, levofloxacin alone, and in combination, against: (A) *Acinetobacter baumannii* type strain ATCC19606; (B) a colistin-resistant strain AB19606R; (C) a colistin-susceptible clinical isolate GN0624; and (D) a colistin-resistant clinical isolate GN1115. CST = colistin; LVX = levofloxacin; Nil = no antibiotic.

Table 2 Logarithmic and fold changes of time-kill experiments at 24 hours in colistin–levofloxacin combinations.

Acinetobacter Baumannii isolates	CST			LVX			CST–LVX		
	LogΔ (CFU/mL)	Fold change	Antibiotic activity	LogΔ (CFU/mL)	Fold change	Antibiotic activity	Log (CFU/mL)	Fold change	Antibiotic activity
ATCC19606	−1.0	10	Growth	−3.5	3162	Bactericidal	0.7	5.0	Growth
AB19606R	+0.6	4.0	Growth	−3.4	2511.9	Bactericidal	≤−3.5[a]	≥3162	Bactericidal no regrowth
GN0624	+1.2	15.8	Growth	−0.4	2.5	Growth	≤−3.5[a]	≥3162	Bactericidal no regrowth
GN1115	+1.8	63.1	Growth	+0.7	5.0	Growth	+1.8	63.1	Growth

[a] Maximum log reduction in CFU/mL detectable by assay (standard inoculum) 5.5 $\log_{10}$ − (lower limit of detection) 2.0 $\log_{10}$ × 0007.
CST = colistin; LVX = levofloxacin.

MPCs and MPIs calculations

The MIC and MPC data and the mutant prevention index (MPI = MPC/MIC) for the two CSTs strains (ATCC19606 and GN0624) are shown in Table 3. The MPCs of CST were very high in the two tested strains (MPCs > 128 mg/L) with CST monotherapy. When 2 mg/L LVX was used in combination with CST; however, a decrease in MPCs of CST could be found (MPCs 16mg/L and 16 mg/L, respectively). Besides, the MPI for CST tested alone was at least 128 for the two isolates. When CST was combined with LVX, nevertheless, the CST MPIs decreased > eight-fold and > 16-fold for the strains of ATCC19606 and GN0624, respectively.

Activities of CST and LVX in the *G. mellonella* infection model

With the live bacterial inocula, larval survival was affected by the inoculum dose and larger doses of bacteria gave reduced larval survival in a dose-dependent manner during a 96-hour incubation. Kinetics of four tested strains at various numbers of CFU/mL in *G. mellonella* over 96 hours are shown in Figure 2. No macroscopic changes or deaths were observed in the untreated group (PBS only), uninfected group (antimicrobial agents only), and non-injection group. Most of the killing occurred in the first 24 hours, followed by further killing in the subsequent 3 days. The optimal inoculum able to promote staggered killing of 80% of larvae over 96 hours for use in treatment assays varied from 10^5 CFU/larval to 10^6 CFU/larval. In the study, CST monotherapy protected *G. mellonella* from *A. baumannii* ATCC19606-mediated killing but performed poorly against the other three strains. LVX had a weak effect in treating the strains of *A. baumannii* ATCC19606 and AB19606R infections, as would be predicted from the *in vitro* susceptibility data. For the clinical strains, however, LVX had no effect on larval survival compared with the PBS-treated controls. Treatment with CST-LVX resulted in survival of 75%, 37.5%, 87.5%, and 37.5% of ATCC19606-, AB19606R-, GN0624-, and GN1115-infected larvae (Figures 3A–D), respectively. The combination was significantly more effective ($p < 0.05$) than CST monotherapy against the CSTs clinical isolates(GN0624) studied *in vivo*.

Discussion

Current treatment options for infections caused by *A. baumannii* are severely limited. CST (polymyxin E derivative), a previously abandoned polymyxin antibiotic, has re-emerged as a last-resort therapeutic option. CST in both parenteral and aerolized administration is, nowadays, the treatment of choice for infections by these species. However, CST administration alone is also associated with significant nephrotoxicity and heteroresistance to CST and is increasingly reported in both type strains and clinical isolates.[2,24] The erosion of effective treatments by resistance, combined with a drug

Table 3 Minimum inhibitory concentrations, mutant prevention concentrations, and mutant prevention index for colistin alone and in combination with levofloxacin.

Acinetobacter baumannii isolate	MICs (mg/L)		MPCs (mg/L)		MPIs (MPC/MIC)	
	CST	CST + LVX[a]	CST	CST + LVX[b]	CST	CST + LVX[c]
ATCC 19606	0.5	0.03125	>128	16	>256	32
GN0654	2	0.5	>256	16	>128	8

[a] MIC of colistin in combination with levofloxacin.
[b] MPC of colistin in combination with 2 mg/L levofloxacin.
[c] MPI colistin in combination with levofloxacin.
CST = colistin; LVX = levofloxacin; MPI (MPC/MIC) = mutant prevention index (mutant prevention concentrations/minimum inhibitory concentrations).

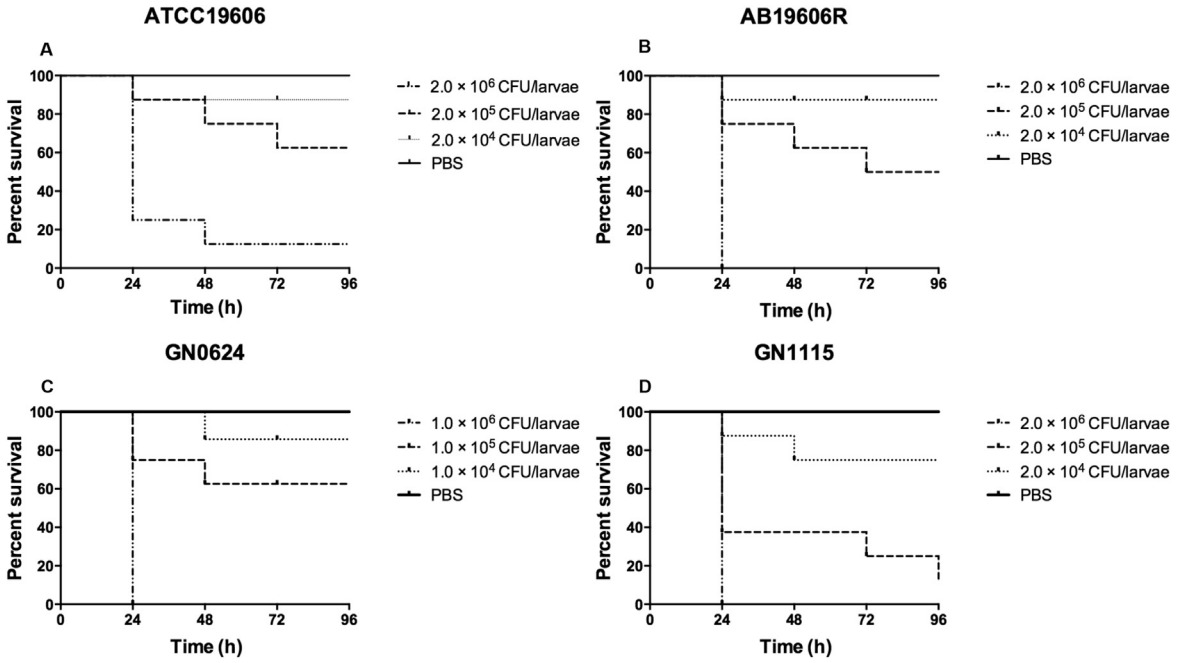

Figure 2. Kill kinetics of strains: (A) ATCC19606; (B) AB19606R; (C) GN0624; and (D) GN1115 at various numbers of CFU/mL in *Galleria mellonella* over 96 hours. Curves were plotted from single experiments using eight insect larvae. PBS = phosphate-buffered saline.

development pipeline that is almost dry, has renewed interest in using unorthodox combination therapies for *A. baumannii* infections.[12]

The bactericidal effect of several major types of antibiotics has recently been demonstrated to be dependent on the formation of toxic amounts of hydroxyl radicals (OH•)

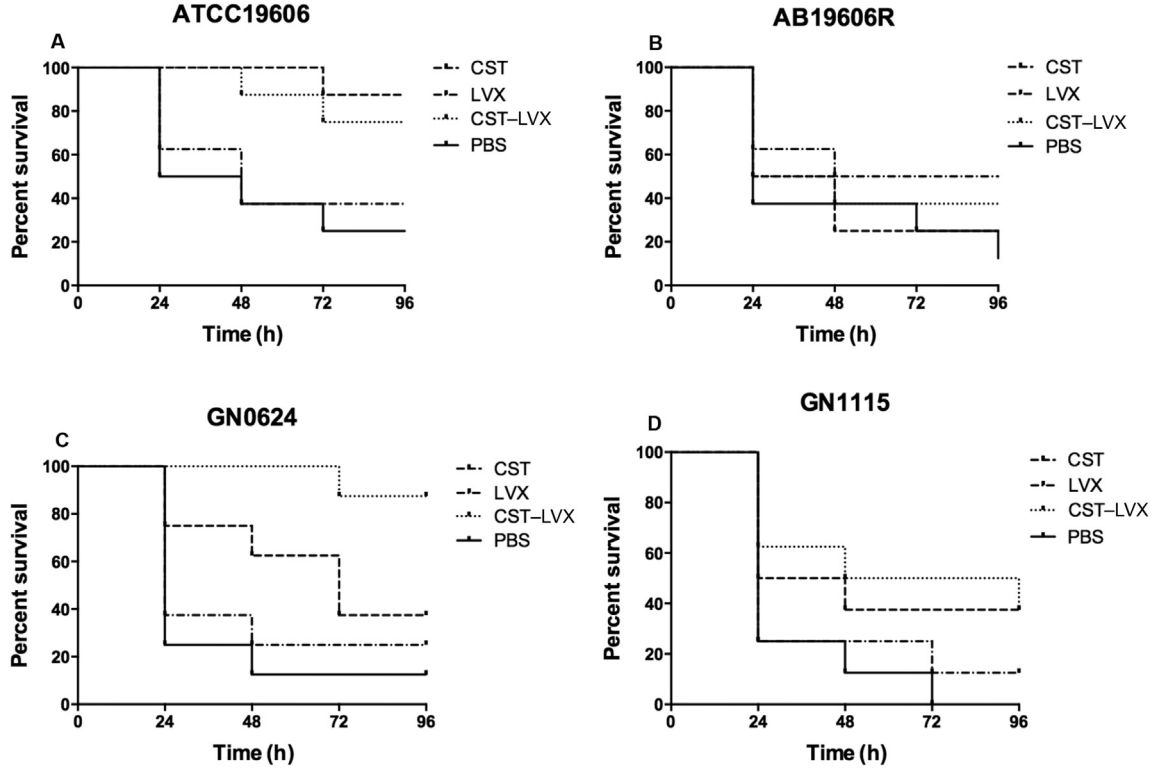

Figure 3. Survival curves for *Galleria mellonella* larvae inoculated with: (A) *Acinetobacter baumannii* type strain ATCC 19606; (B) a colistin-resistant *A. baumannii* strain (AB19606R); (C) two clinical strains GN0624; and (D) GN1115 following treatment with colistin (2.5 mg/kg), levofloxacin (6.7 mg/kg), or a levofloxacin—colistin combination. Data from a single representative experiment. CST = colistin; LVX = levofloxacin; PBS = phosphate-buffered saline.

resulting from oxidative stress in metabolically active cells. However, bactericidal activity of CST does not follow the common OH•-mediated killing discovered in major groups of bactericidal antibiotics, including quinolones.[25] Moreover, combination of CST and ciprofloxacin (fluoroquinolones) has been used with clinical success for decades at the Copenhagen CF Center for preventing and postponing chronic *P. aeruginos* infection.[26] As we know, fluoroquinolones exhibit concentration-dependent killing and a postantibiotic effect. Based on these data, we focus on LVX which is another outstanding representative of the third generation of quinolone antibiotics. LVX has been a useful broad-spectrum antimicrobial in the past few decades, whereas the efficacy of this agent may be now fading. Therefore, we looked at the effects of combining the CST with LVX against *A. baumannii* in this study.

In the current study, LVX lacked *in vitro* activity as a single agent against the two clinical isolates. Nevertheless, we confirmed that the combination of CST and LVX exhibited enhanced *in vitro* and *in vivo* activity against the CSTs clinical isolate, in accordance with the previous results reported by Safarika et al.[6] The bactericidal activity of CST is partly due to its detergent effect on the bacterial cell membrane. This disruptive effect on membrane integrity may account for the *in vitro* synergy observed with LVX.[27] The presented results of chequerboard assays provide evidence that CST possess a considerable synergy with LVX against CSTs *A. baumannii*. The efficacy of the interaction with LVX is greater as the MIC levels of CST and LVX decrease. The SBPI is a novel parameter that relates the magnitude of the interaction to the pharmacodynamic breakpoints used to determine susceptibility *in vivo*. A SBPI of > 2 indicates that the agents are more active in combination than when used alone.[13] In time—kill assays, the clinical isolate (CSTs) did not display sustained bactericidal activity at susceptible breakpoint concentration with CST monotherapy due to its heteroresistance as described previously.[28] When combined with LVX, however, the drugs were not only synergistic but also bactericidal and prevented the regrowth of CSTs bacteria. Furthermore, the concentrations of CST required to mediate LVX synergy *in vitro* are relatively low, which may reduce the risk of developing renal impairment if the agents are given together. Yet despite all that, this combination had poor antibiotic activities on the CSTr strains.

A. baumannii has the ability to form biofilms, which may play a role in the process of colonization. The ability to adhere to and form biofilms on the surface of inanimate objects might be the cause of its strong ability to survive in hospital environments.[29] Low O_2 availability is a common condition in infectious biofilm. As far as we know, bactericidal drugs such as CST with OH•-independent killing have a better effect on anaerobically compared with aerobically grown cells. Therefore, antimicrobial peptides may be useful for the treatment of Gram-negative bacteria growing at low oxygen tension.[26] However, CST monotherapy seemed unlikely to make much effect in our study. However, the synergistic effect of LVX—CST combination therapy was observed both in CSTs and CSTr strains. The results indicated that the LVX—CST combination had beneficial effect *in vitro* biofilm infections.

Besides simply focusing on enhancing the activity of bacterial killing, evaluation of combination therapy on resistance inhibiting is also extremely important as drug-resistant strains are emerging so rapidly. The problem of antibiotic resistance is likely to get worse, in part because the therapeutic concentrations currently used, which block growth of the majority of the susceptible pathogens, are often the very concentrations required to selectively enrich the resistant mutant portion of the population.[30] In our experiment, although the MICs of CST to ATCC19606 and GN0624 were low, the MPCs of this agent were high. The high MPC values in our study suggest the possibility of enriching CST resistant mutant subpopulations during treatment with CST monotherapy. Zhao and Drlica[21] showed that with antibiotics individually having high MPCs, combination therapy provided a feasible method to curb the emergence of resistance. Combination therapy using two drugs acting with different antimicrobial mechanisms would require susceptible bacteria to acquire two concurrent mutations for growth. Our experimental results demonstrated that combinations of CST and LVX could prevent the emergence of CSTr *A. baumanni*, which was consistent with the previous results reported by Cai et al.[31]

The use of *G. mellonella* as a model to elucidate the pathogenicity of microorganisms has been widely reported.[32] It shares many of the advantages of mammalian models while being free of the ethical and logistical constraints that accompany their use. *In vitro* data on the potential for CST-LVX regimens to be beneficial in treatment were correlated by the *in vivo* studies using *G. mellonella* larvae. In *Galleria* organisms, CST and LVX given together at humanized doses significantly improved survival against infections with *A. baumannii*. The main consideration of using the minimum administered dose for CST is to reduce its nephrotoxicity. Our *in vivo* model demonstrated improvement with addition of CST (2.5 mg/kg) to LVX (6.7 mg/kg). The precise mechanisms of the synergy of bacterial killing and suppression of resistance for CST and second antibiotics are largely unknown but might result from immunomodulatory activities of the compound. Whether this phenomenon is species-specific and has any implications for the selection of fluoroquinolones for use in unorthodox combinations to treat human infections is unknown and requires further investigation.

In summary, a synergistic or additive effect between CST and LVX was observed *in vitro* and *in vivo* against CSTs *A. baumannii* strains, although not against CSTr ones. However, our results should be considered with care for several reasons. A limitation of this study is the use of limited isolates. In addition, we cannot conclude that our *in vitro* results will hold true with treatment durations longer than 72 hours. Besides, for LVX—CST combination therapy to be employed effectively and safely, further work on the pharmacokinetic parameters of this combination *in vivo* will be needed. Moreover, studies employing mammalian models that seek to assess the activity of CST combinations *in vivo* versus Gram-negative pathogens are now warranted. Finally, confirmation of these results in clinical studies is needed before these regimens can be adopted for use in the care of patients.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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