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Short Communication

Synergistic activity between colistin and the ionic liquids 1-methyl-3-dodecylimidazolium bromide, 1-dodecyl-1-methylpyrrolidinium bromide, or 1-dodecyl-1-methylpiperidinium bromide against Gram-negative bacteria

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ABSTRACT

Objectives: Ionic liquids have shown potential for applications as antimicrobials. Their antimicrobial activity has been shown to be higher against Gram-positive than Gram-negative bacteria, suggesting a protective role for the outer membrane of Gram-negative microorganisms. Colistin is a last-resort antibiotic often used for treating infections caused by multi-drug resistant Gram-negative bacteria. Colistin interacts with the bacterial lipopolysaccharide, thus altering the structure and increasing the permeability of the outer membrane. The aim of this study was to investigate the interaction between colistin and the ionic liquids 1-methyl-3-dodecylimidazolium bromide, 1-dodecyl-1-methylpyrrolidinium bromide, and 1-dodecyl-1-methylpiperidinium bromide against Gram-negative bacteria of clinical importance such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.
Methods: The interaction between colistin and ionic liquids against Gram-negative bacteria was evaluated by the checkerboard assay. Bacterial killing assays against *P. aeruginosa* were carried out to assess whether the synergistic combinations were bactericidal.
Results: The results of checkerboard assays showed that all three ionic liquids interacted synergistically with colistin against *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* but not against *E. coli*, which was more sensitive to all three ionic liquids compared with the other tested species. The synergistic combinations showed no haemolytic activity. Bacterial killing assays showed that the synergistic effect between colistin and each one of the three tested ionic liquids against *P. aeruginosa* was bactericidal.
Conclusion: Overall, the results obtained suggest that colistin and ionic liquids might be used in combination for possible applications to combat infections caused by multi-drug resistant Gram-negative bacteria.

1. Introduction

Ionic liquids (ILs) have shown potential for applications in different fields of medical sciences [1]. In particular, several reports have documented the antimicrobial activity of several ionic liquids, suggesting their possible applications as antimicrobial agents [2–8].

In a previous study [7], a panel of ILs were comparatively evaluated for their antimicrobial activity against Gram-positive and Gram-negative bacteria, and for their toxicity to human red blood cells. The results showed that the ILs 1-methyl-3-dodecylimidazolium bromide (IL 1), 1-dodecyl-1-methylpyrrolidinium bromide (IL 2), and 1-dodecyl-1-methylpiperidinium bromide (IL 3) were characterized by high antimicrobial potency and relatively low haemolytic activity. In general, the antimicrobial activity was higher for Gram-positive than for Gram-negative bacteria, suggesting that the outer membrane of Gram-negative microorganisms may represent a physical barrier to the lytic activity of ionic liquids.

In 2017, Hanna et al. [9] reported that both IL 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium

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tetrafluoroborate synergistically enhance the permeation of lipid bilayer vesicles by polymyxin B, leading to lipid membrane disruption, and suggested that ILs could act synergistically as antibacterial agents in combination with membrane-disrupting antibiotic compounds.

Colistin is a last-resort antibiotic that is often used for treating infections sustained by multi-drug resistant (MDR) Gram-negative bacteria [10], which are increasingly responsible for hospital-acquired bloodstream infections and represent a major global public health concern [11,12]. Unfortunately, MDR Gram-negative bacterial strains characterized by plasmid-mediated colistin resistance have been recently isolated with increasing frequency [13]. Nevertheless, colistin remains one of the few treatment options for infections caused by Gram-negative strains with resistance to most or all other classes of antimicrobial drugs [14].

The present study aimed at evaluating possible synergistic effects between colistin and ILs against Gram-negative bacteria, including members of the Enterobacteriaceae family, namely *Escherichia coli* and *Klebsiella pneumoniae*, and non-fermenting Gram-negative species, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

2. Material and methods

2.1. Bacterial strains and culture conditions

E. coli (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *K. pneumoniae* (ATCC BAA-1705) were purchased from the American Type Culture Collection. *A. baumannii*, clinical isolate AB01, was isolated from a patient admitted to the Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy); this strain was resistant to ciprofloxacin (MIC < 2 μg/mL), doripemen (MIC < 8 μg/mL), gentamicin (MIC < 4 μg/mL), imipenem (MIC < 16 μg/mL), meropenem (MIC 64 μg/mL) and trimethoprim/sulfamethoxazole (MIC < 4 μg/mL), and susceptible to amikacin (MIC 8 μg/mL). All the bacterial strains used in this study were propagated in Mueller–Hinton broth (Oxoid, Thermo Fisher Scientific Inc., Basingstoke, UK), harvested by centrifugation (4500 rpm, 10 min, 25 °C), resuspended in 1/10 vol of fresh medium supplemented with 20% (v/v) glycerol, and stored in aliquots at −80 °C. Before use for experiments, bacterial strains were plated onto Mueller–Hinton agar, and cultures were incubated for 24 h at 37 °C.

Colistin sulfate (Acros Organics, Thermo Fisher Scientific Inc.) was stored at 4 °C in the dark; stock solutions of 10 mg/mL colistin sulfate were prepared in phosphate buffer saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), sterile filtered, and stored in aliquots at −20 °C.

2.2. Preparation of ionic liquids 1–3

Ionic liquids 1–3 were prepared pure, as previously described [7]. Briefly, 1-bromododecane was added to the selected *N*-nucleophile (*N*-methyl-imidazole, *N*-methyl-pyrrolidine, *N*-methyl-piperidine) in CH_3CN and the reaction mixture was heated to 80 °C. After 18 h, the reaction solvent was removed under reduced pressure and the reaction product was recovered by recrystallization from dry acetonitrile/toluene.

2.3. Checkerboard assay

The interaction between colistin and IL 1, IL 2 or IL 3 against *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* was evaluated by a checkerboard titration method using 96-well round bottom polystyrene microtitre plates as previously described [15]. This assay was performed in Mueller–Hinton broth in a volume of 200 μL. Serial dilutions of IL/colistin, alone or in combination, were prepared in 100 μL Mueller–Hinton broth at 2× their final concentration; then, 100 μL of mid-log phase bacterial suspension in Mueller–Hinton broth was added at a final density of about 2×10^5 CFU/mL. Each plate included sterility control wells, containing the medium alone. Next, the plates were incubated for 24 h at 37 °C before reading the minimum inhibitory concentration (MIC) of colistin and IL, alone or in combination, defined as the lowest concentration of the agent that produced complete inhibition of visible bacterial growth. A variability of one dilution was considered acceptable to determine the MIC of colistin and/or IL 1–3. The fractional inhibitory concentration (FIC) index for the combinations was determined applying the following formula: FIC index = (MIC of colistin in combination)/(MIC of colistin alone) + (MIC of IL in combination)/(MIC of IL alone). FIC indices were interpreted as follows: ≤0.5 as synergy, >0.5 to ≤4 as indifference, and >4 as antagonism. FIC index values reported in this study were the lowest FIC indices observed in at least three independent experiments.

2.4. Bacterial killing assay

P. aeruginosa was inoculated from stationary phase culture into Muller-Hinton broth and incubated at 37 °C with agitation until mid-log phase. Then, bacterial cells were harvested by centrifugation (13 000 rpm, 5 min, 25 °C), washed once with PBS, pH 7.4, and resuspended into an equal volume of sterile PBS. The bacterial suspension was diluted in PBS to a density of about 2×10^6 CFU/mL. One hundred microlitres of diluted bacterial suspension were added to 100 μL of colistin and/or IL 1–3 at 2× their final concentrations in PBS, and then incubated for 90 min at 37 °C with agitation (160 rpm) before plating serial two-fold dilutions onto Mueller–Hinton agar for CFU/mL determination. As positive control, 100 μL of bacterial suspension was added to 100 μL of PBS, and 100 μL of serial dilutions were plated onto Mueller–Hinton agar plates at time 0 and after 90 min incubation for CFU counts.

The minimum bactericidal concentration (MBC) was defined as the minimum concentration of colistin/IL 1–3 causing a decrease in CFU/mL of ≥3 log after 90 min incubation.

Synergy was defined as a decrease in CFU/mL of ≥2 log by the combination of colistin and IL in comparison with the most active constituent. The results are reported as mean log CFU/mL ± SEM of at least four independent experiments.

Table 1
Minimum inhibitory concentration (MIC) of colistin and IL 1–3 against *E. coli*,*P. aeruginosa*, *K. pneumoniae* and *A. baumannii*.

Antimicrobial	MIC (μg/mL) against			
	Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*
IL 1	20	160	80	80
IL 2	80	320	160	320
IL 3	40	320	160	320
Colistin	2	1	2	4

2.5. Haemolysis assay

A haemolysis assay was used to evaluate the cytotoxicity of colistin and IL 1, IL 2 or IL 3 alone and in combination. Briefly, blood from three healthy individuals was collected in vacuum tubes containing K_3EDTA (Becton Dickinson & Co, Milan, Italy) as anticoagulant. Red blood cells (RBCs) were harvested by centrifugation at 800 × *g* for 5 min at room temperature, washed three times with PBS, added to 0.25% (v/v) dimethyl-sulfoxide (DMSO) and resuspended in PBS/0.25% DMSO to a concentration of 8% (v/v). An aliquot (100 μL) of this suspension was transferred into each well of a round-bottom 96-well microtitre plate (Corning Costar, New York, NY) and mixed 1:1 with 100 μL of IL 1–3 or colistin solution at twice the desired concentration or 50 μL of 4 × IL 1–3 and colistin for synergy combinations. Each concentration or combination was tested in triplicate for each test.

After incubation for 1 h at 37 °C, the microtitre plate was centrifuged (800 × *g*, 5 min) and 100 μL of the supernatants were transferred to a flat-bottom 96-well plate (Sarsted, Milan, Italy) for measurement of the haemoglobin release by reading the absorbance at 450 nm. Data were normalized between the 0% haemolysis of RBCs in PBS/DMSO and 100% haemolysis of RBCs in PBS/DMSO/1% Triton X-100. The percentage of haemolysis was calculated by the following formula: $(A_{colistin/IL1-3} - A_{PBS/DMSO})/(A_{Triton\ X\text{-}100} - A_{PBS/DMSO}) \times 100\%$.

The three blood samples were withdrawn from healthy volunteers, casually chosen among the authors of this manuscript, to perform the haemolysis assay. The local ethical committee ruled that no notification was necessary in this case.

2.6. Statistical analysis

Results were evaluated by one-way analysis of variance (ANOVA). Differences in CFU/mL between the various treatments in bacterial killing assays were evaluated with the Tukey-Kramer test. The significance threshold was set at a *P*-value of 0.05.

3. Results

3.1. Interaction between colistin and IL 1–3 against Gram-negative bacteria in checkerboard assays

First, the MIC of colistin and ILs alone were determined against the *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* strains used in this study (Table 1). The MIC values of IL 1–3 against *E. coli* and *P. aeruginosa* were consistent with those previously reported by Florio et al. [7], whereas the MICs against *K. pneumoniae* and *A. baumannii* had not been previously reported. Then, colistin and ILs were tested in different combinations of concentrations using the checkerboard assay. As reported in Table 2, IL 1, IL 2, and IL 3 showed a synergistic effect (FIC ≤0.5) with colistin against *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, but not against *E. coli*, for which observed FIC values were >0.5 and <4, indicating indifference. A variability of one two-fold dilution in MIC values was observed for *K. pneumoniae* and *A. baumannii* in replicates of experiments. However, this variability did not affect the overall interpretation of results, since all observed FIC values were ≤0.5 (synergy).

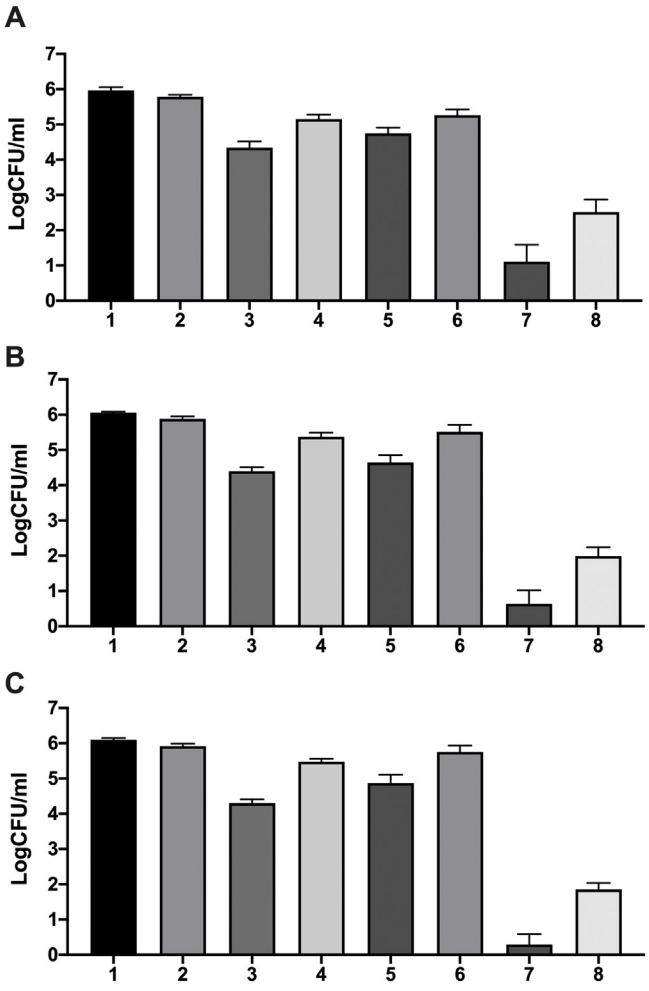

Fig. 1. Synergistic activity between colistin and ionic liquid (IL) 1 (A), IL 2 (B) or IL 3 (C) against *P. aeruginosa* in killing assays. Columns 1 and 2: positive controls (bacteria incubated in PBS) at time 0 and after 90 min incubation, respectively; columns 3 and 4: 0.5× minimum bactericidal concentration (MBC) (2 μg/mL) and 0.25 × MBC (1 μg/mL) colistin, respectively; columns 5 and 6: 0.5 × MBC (10 μg/mL for IL 1, 40 μg/mL for IL 2 and IL 3) and 0.25 × MBC (5 μg/mL for IL 1, 20 μg/mL for IL 2 and IL 3) IL 1–3, respectively; column 7: 0.5 × MBC colistin + 0.5 × MBC IL 1–3; column 8: 0.25 × MBC colistin + 0.25 × MBC IL 1–3.

Table 2
FIC values observed in checkerboard assays for the different combinations of ionic liquid/colistin.

Ionic liquid	FIC[a] indices of the combinations colistin/ionic liquid against			
	Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*
1	0.625 (0.25/10; 1/2.5)[b]	0.375 (0.25/20; 0.125/40)	0.25 (0.25/10) to 0.375 (0.5/10; 0.25/20)	0.5 (1/20)
2	0.75 (1/20; 0.5/40)	0.5 (0.25/80)	0.375 (0.5/20; 0.25/40) to 0.5 (0.5/40)	0.375 (0.5/80) to 0.5 (1–80)
3	0.75 (0.5/20; 1/10)	0.5 (0.25/80)	0.375 (0.5/20; 0.25/40) to 0.5 (0.5/40)	0.375 (0.5/80) to 0.5 (1/80)

[a] FIC: fractionary inhibitory concentration. FIC values ≤0.5 indicate synergy; values <0.5 and ≤4 indicate indifference, and values >4 indicate antagonism.
[b] Numbers in parentheses indicate MIC values of colistin/ionic liquid in the combinations.

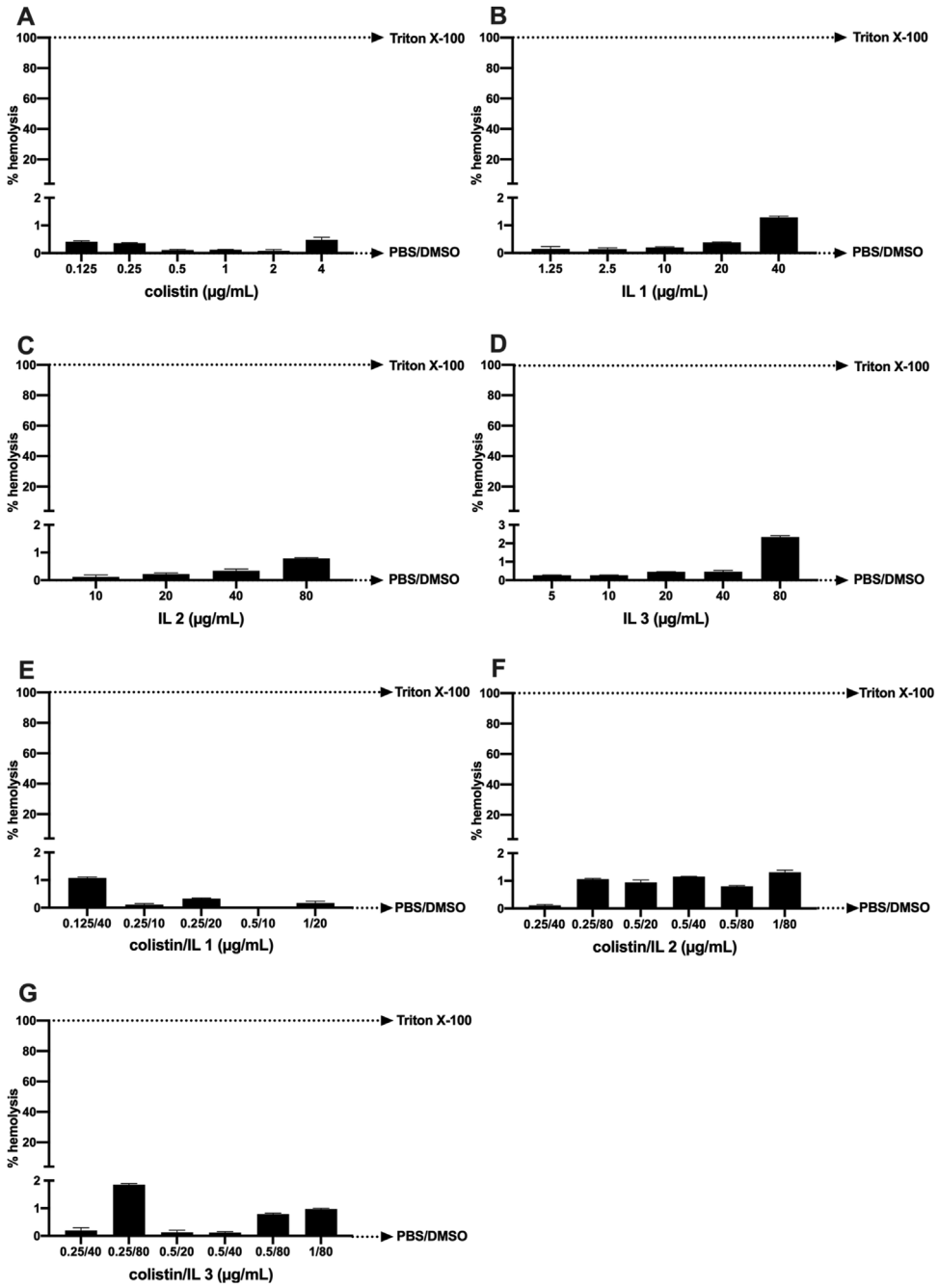

Fig. 2. (A–G). Haemolytic activity of IL 1–3 and/or colistin at various concentrations. IL 1–3 and/or colistin were incubated with 8% RBC suspension. The tested concentrations of the combinations are reported as x/y, where x and y represent the concentrations of colistin and IL 1–3, respectively. The results are expressed as mean percentage of haemolysis. RBCs incubated with 1% Triton X-100 and PBS/DMSO (untreated) were considered as 100% and 0% haemolysis, respectively. The percentage of haemolysis was calculated as follows: $(A_{\text{colistin and/or IL 1–3}} - A_{\text{PBS/DMSO}})/(A_{\text{Triton X-100}} - A_{\text{PBS/DMSO}}) \times 100\% \pm \text{SEM}$ from three independent experiments.

3.2. Synergistic activity between colistin and IL 1–3 in bacterial killing assays

In order to assess whether the synergistic activity of the combinations of colistin and IL 1–3 was bactericidal, non-bactericidal concentrations of colistin and IL 1–3 were tested in bacterial killing assays against *P. aeruginosa*. First, the MBCs of colistin and IL 1–3 against *P. aeruginosa* were determined in the same experimental conditions used to test the various combinations. The MBC results after 90 min incubation at 37 °C were: colistin, 4 μg/mL; IL 1, 20 μg/mL; IL 2 and IL 3, 80 μg/mL. The MBCs of IL 1–3 against *P. aeruginosa* coincided with those previously reported [7]. Then, colistin and IL 1–3 were tested, alone or in combination, at non-bactericidal concentrations (0.5× and 0.25× MBC) as shown in Fig. 1. MBCs of colistin and IL alone were also included in each experiment as internal controls to ascertain that they were bactericidal (data not shown). The results revealed that all the combinations tested of colistin and IL 1, IL 2 or IL 3 synergistically killed *P. aeruginosa*, with ≥2 log reduction of CFU/mL in comparison with the most active constituent in each experiment. All the tested combinations were also bactericidal, showing ≥3 mean log reduction of CFU/mL compared with the untreated control (bacteria incubated in PBS for 90 min).

3.3. Haemolytic activity of IL 1–3 and/or colistin

A haemolysis assay was performed to evaluate possible toxic effects of IL 1–3 and/or colistin on human red blood cells (Fig. 2). The results revealed that colistin alone had no haemolytic activity (<2%) until the highest MIC value (4 μg/mL) (Fig. 2A). In a previous study, IL 1–3 did not show haemolysis at concentrations corresponding to their MIC and 10× MIC (2.5–25 μg/mL, 10–100 μg/mL and 5–50 μg/mL, respectively) [7]. In the present study, a haemolysis assay was performed for all the IL 1–3 and colistin concentrations for which a synergistic effect was obtained (as shown in Table 2). As expected, IL 1, IL 2 or IL 3 alone showed no haemolytic activity (<2%) at all the tested concentrations, with the exception of IL 3, which exhibited 2.34% haemolysis at 80 μg/mL (Fig. 2B–D). All the IL 1–3 and colistin combinations tested with FIC value <0.5 showed no haemolytic activity (<2%) (Fig. 2E–G).

4. Discussion

The central finding of this study is that colistin and ionic liquids can act synergistically against different species of Gram-negative bacteria. The possible synergistic antimicrobial effect between colistin and ILs had been previously hypothesized by Hanna et al. [9] who showed that the ILs 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium tetrafluoroborate could enhance the permeation of lipid bilayer vesicles by polymyxin B, which is a polypeptide antibiotic of the same class as colistin. The present study provides the first direct evidence of a synergistic effect between colistin and IL 1, IL 2 or IL 3 against Gram-negative bacteria.

In a previous study comparing the antimicrobial activity of different classes of ionic liquids, ILs 1–3 were selected for their potent antibacterial activity and relatively low toxicity to human blood cells [7]. One of the findings of that study was the higher sensitivity to ionic liquids by Gram-positive compared with Gram-negative bacteria. Therefore, it was hypothesized that the outer membrane of Gram-negative bacteria might represent a protective barrier against the antimicrobial activity of ILs. Since colistin affects the structure and increases the permeability of the outer membrane of Gram-negative bacteria after binding to lipopolysaccharide [16], we considered the possibility that colistin could favour the entrance of ILs through this barrier, thus allowing them to exert their antimicrobial activity. Indeed, the results of the present study by the checkerboard assay showed a synergistic effect between colistin and IL 1, IL 2 or IL 3 against *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, which supports our hypothesis. However, synergy between colistin and ILs was not observed against *E. coli*. The lack of synergistic effect against *E. coli* might be due to the higher sensitivity of this microorganism to ILs, compared with that observed for *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, for which MIC values of IL 1–3 were higher. In particular, the higher sensitivity to ILs of *E. coli* compared with the other tested Gram-negative species might minimize the advantage of outer membrane permeation by colistin in favouring ILs activity.

The results of the killing assays on *P. aeruginosa* showed that the synergistic effect of colistin and ionic liquids was bactericidal, reinforcing the hypothesis that colistin might favour the penetration of ILs through the outer membrane of this microorganism, and allow them to exert their bactericidal activity on the inner membrane and/or other possible bacterial targets.

It is worth mentioning that there is an open debate on the toxicity of ionic liquids. Indeed, some of them displayed remarkable cytotoxicity [17] while others were completely safe [18]. It is difficult to make generalization regarding the properties of ionic liquids, and previous attempts have often been proved wrong, due to the large number of possible combinations of anions and cations [19] and the availability of only a small set of safety data. Indeed, even modest structural changes can lead to markedly different toxicity profiles [20].

Noteworthy, the results obtained in this study showed no haemolytic activity (<2%) for the administration of colistin alone or in combination with ILs 1–3 when used in the concentration range of interest, namely where the synergistic effect was observed. Further analysis will be needed to assess whether or not longer exposure times have the potential to show a cytotoxic effect.

5. Conclusions

Overall, the results of this study demonstrate a synergistic effect between colistin and IL 1–3 against *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, thus suggesting possible applications of these compounds in combination as antimicrobials. Further studies will be needed to investigate the best possible ways to employ colistin and ILs in combination to combat difficult-to-treat, hospital-acquired infections sustained by MDR Gram-negative bacteria.

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Contributors

WF, SB, LG, FD and AL conceived the study and contributed to the experimental design; WF, CR, SB, FD and LG carried out the experiments; WF and FD wrote a draft of the manuscript; AL and LG critically revised the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

None declared.

Ethical approval

This study was approved by the local ethical committee, Comitato Etico di Area Vasta Nord-Ovest, University of Pisa, and conducted in full accordance with the principles of the Declaration of Helsinki. Samples were taken as part of the standard patient care, anonymized by the clinical personnel, and used anonymously by the Research personnel. According to the local ethical committee, for this type of study, formal consent to participate was not required (Prot. N. 32634).

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