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ORIGINAL ARTICLE

Synergistic activity of synthetic N-terminal peptide of human lactoferrin in combination with various antibiotics against carbapenem-resistant *Klebsiella pneumoniae* strains

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Abstract The spread of multi-drug resistant (MDR) *Klebsiella pneumoniae* strains producing carbapenemases points to a pressing need for new antibacterial agents. To this end, the in-vitro antibacterial activity of a synthetic N-terminal peptide of human lactoferrin, further referred to as hLF1–11, was evaluated against *K. pneumoniae* strains harboring different carbapenemase genes (i.e. OXA-48, KPC-2, KPC-3, VIM-1), with different susceptibility to colistin and other antibiotics, alone or in combination with conventional antibiotics (gentamicin, tigecycline, rifampicin, clindamycin, and clarithromycin). An antimicrobial peptide susceptibility assay was used to assess the bactericidal activity of hLF1–11 against the different *K. pneumoniae* strains tested. The synergistic activity was evaluated by a checkerboard titration method, and the fractional inhibitory concentration (FIC) index was calculated for the various combinations. hLF1–11 was more efficient in killing a *K. pneumoniae* strain susceptible to most antimicrobials (including colistin) than a colistin-susceptible strain and a colistin-resistant MDR *K. pneumoniae* strain. In addition, hLF1–11 exhibited a synergistic effect with the tested antibiotics against MDR *K. pneumoniae* strains. The results of this study indicate that resistance to hLF1–11 and colistin are not strictly associated, and suggest an hLF1–11-induced sensitizing effect of *K. pneumoniae* to antibiotics, especially to hydrophobic antibiotics, which are normally not effective on Gram-negative bacteria. Altogether, these data indicate that hLF1–11 in combination with antibiotics is a promising candidate to treat infections caused by MDR-*K. pneumoniae* strains.

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Introduction

The worldwide spread of carbapenem-resistant *Klebsiella pneumoniae* strains represents a major concern for public health [1]. Carbapenem resistance is mostly due to the production of carbapenemases that hydrolyze all β-lactam antibiotics including carbapenems, which are considered one of the last resorts for treating infections caused by *Enterobacteriaceae* [2–4]. Carbapenemases have been classified into three different molecular classes: Amber class A (such as KPC type), class B (such as VIM type), and class D (such as OXA-48 type). All of these are widely distributed among *Enterobacteriaceae*, particularly in *K. pneumoniae* strains associated with systemic infections [5, 6]. Moreover, strains harboring KPC-type genes of class A are often resistant to several other classes of drugs, including fluoroquinolones and aminoglycosides [6, 7]. Therefore, the therapeutic options to treat infections caused by such MDR strains are very

limited, and high rates of mortality are recorded [8–10]. Among the few drugs that are still effective, colistin, tigecycline, gentamicin, and fosfomycin are often used in combination therapy [11, 12], yet *K. pneumoniae* strains resistant to these drugs have been recently isolated [13, 14]. It should be noted that development of novel antimicrobial agents against Gram-negative bacteria is also very limited [8, 15].

In this context, cationic antimicrobial peptides (AMP) have gained attention as possible new therapeutic candidates. A generally accepted mechanism of action of antimicrobial peptides involves electrostatic binding between the positively charged peptides and negatively charged structural elements of bacteria, e.g., lipopolysaccharide [16, 17]. Lactoferrin (LF) is a 77 kDa iron-binding multifunctional glycoprotein. It is present in the milk of humans and other mammals, in the blood plasma and neutrophil-specific granules, and is one of the major components of virtually all exocrine secretions of mammals, such as saliva, bile, tears, and pancreatic juice. Its functions include the regulation of innate immunity, iron transfer to cells, control of the level of free iron in blood, interaction with nucleic acids, heparin, and polysaccharides, and pronounced antimicrobial and antiviral activities [18, 19]. Importantly, lactoferrin is a source of peptides with antimicrobial activity. Human LF generates, by acid-pepsinolysis, the antimicrobial peptide lactoferricin H (residues 1 to 47), which contains two cationic domains (residues 2 to 5 and 28 to 31). A synthetic peptide comprising the first cationic domain of lactoferricin H, further referred to as hLF1–11, exerts effective antibacterial and antifungal activities, as demonstrated by in-vitro and in-vivo studies in systemic infections caused by fluconazole-resistant *Candida albicans*, multidrug-resistant *Staphylococcus aureus*, and *Acinetobacter baumannii* strains [20–26]. Moreover, hLF1–11 exerts modulatory effects on cells of the human immune system [27, 28], which is increasingly recognized as an important contribution to the clearance of infections [29–31].

Several AMP have shown synergistic activity with conventional antibacterial and antifungal agents, and combination therapies have also been proposed as a mean to reduce the frequency of emergence of resistant strains [32–35]. In the present study, we aimed at evaluating the antibacterial activity of hLF1–11 alone or in combination with various antibiotics against carbapenemase-producing *K. pneumoniae* strains harboring different resistance genes.

Materials and methods

Bacteria

Three *K. pneumoniae* strains were isolated from positive blood cultures of patients admitted to the Azienda Ospedaliero–Universitaria Pisana (Pisa, Italy) and selected on the basis of their antimicrobial susceptibility profile: a susceptible *K. pneumoniae* strain, a colistin-sensitive MDR strain, and a colistin-resistant (MIC 16 μg/ml) MDR *K. pneumoniae* strain. Their antimicrobial susceptibility profile was determined by the Vitek 2 system (bioMeriéux, l'Etoile, France) and confirmed by E-test (bioMeriéux) and Sensititre Aris System (Trek Diagnostic Systems, Thermo Fisher Scientific, OH, USA). The antimicrobials tested were the following: amoxicillin/clavulanic acid, ampicillin, carbapenems (imipenem, meropenem, ertapenem), piperacillin/tazobactam, cephalosporins (cefepime, cefotaxime, ceftazidime), aminoglycosides (amikacin, gentamicin, and tobramycin), fluoroquinolones (levofloxacin and ciprofloxacin), colistin, tigecycline, and trimethoprim/sulfamethoxazole. For both MDR *K. pneumoniae* strains, phenotypic and genotypic tests were performed to determine the type of carbapenemase. A combination disk test was used for the phenotypic characterization (Neosensitabs®, Rosco, Taastrup, Denmark). For genotypic characterization, after DNA extraction [36], the presence of bla_{KPC} gene was determined using Hy-KPC real-time PCR, kindly provided by Hy-Labs (Hy-Labs, Israel), with the ABI Prism® 7500 instrument (Applied Biosystems, Foster City, CA, USA). Next, the bla_{KPC} gene fragment (1010 bp) was amplified using KPC-fw/KPC-rv primers [37], purified after 1% agarose gel electrophoresis by 5Prime-Agarose GelExtract mini kit (5 Prime GmbH, Hilden, Germany), and sequenced using cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing apparatus (Eurofins Genomics Ebersberg, Germany). Sequencing results were analyzed by the sequence alignment Geneious software platform [38], and compared with the reference sequence from NC-021660 (*Klebsiella pneumoniae* FCF3SP plasmid, bla_{KPC} type 2).

In addition, four multidrug resistant *K. pneumoniae* strains producing different carbapenemases (OXA-48, KPC-2, KPC-3, and VIM-1) and previously described [39] were used, and a *K. pneumoniae* ATCC® BAA-1705™ was included as a positive control for KPC production. Bacteria were cultured in Luria Bertani (LB) broth (Sigma–Aldrich, St. Louis, MO, USA) to mid-log phase; aliquots of this culture, containing 10^8 colony-forming unit (CFU)/ml, were supplemented with 20% (vol/vol) glycerol and stored at −80 °C. For antimicrobial susceptibility tests and synergy studies, each strain was cultured overnight in LB at 37 °C, and sub-cultured for 2 h on a rotary wheel at 37 °C.

Synthetic peptide and antibiotics

The synthetic peptide corresponding to residues 1–11 (GRRRRSVQWCA) of hLF, further referred to as hLF1–11, was purchased from Peptisyntha (Brussels, Belgium). The purity of this peptide exceeded 99%, as determined by reverse-phase high performance liquid chromatography

(RP-HPLC). Peptide stocks at a concentration of 10 mM in 0.01% acetic acid (pH 3.7) were stored at −20 °C, and diluted to the desired concentration before use. The following antibiotics were tested in synergy studies: rifampicin, clarithromycin, clindamycin, gentamicin, and tigecycline, all purchased from Sigma–Aldrich (St. Louis, MO, USA). Rifampicin and tigecycline were dissolved in dimethyl sulfoxide (Fluka Chemie GmbH, Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands) and stored at −80 °C. The final concentration of DMSO was <0.1%. The other antibiotics were dissolved in sterile distilled water and stored at −20 °C until use. Mueller Hinton (1:16 diluted) and NaPB (10 mM Na-phosphate buffer, pH 7.4) were freshly prepared for each experiment.

Antimicrobial peptide susceptibility assay

To assess the bactericidal activity of hLF1–11, *K. pneumoniae* cells were harvested in mid-log phase by centrifugation at 4,500 × g for 10 min, washed twice to completely remove traces amounts of culture medium (LB broth), and resuspended at a concentration of 10^7 CFU/ml in NaPB. Aliquots of this suspension were mixed with equal volumes of various concentrations of hLF1–11, prepared in NaPB, and incubated for 1 h at 37 °C. Thereafter, the number of viable bacterial cells was determined by plating serial dilutions of each sample on blood agar plates (Becton Dickinson & Co, BD; Milan, Italy).

Synergy studies

Synergy analyses of hLF1–11 and antimicrobial agents were carried out by a checkerboard titration method using 96-well round bottom polystyrene microtiter plates. This assay was performed in MH broth (MHB; Oxoid, Milan, Italy) diluted 1/16 in NaPB, since in preliminary experiments hLF1–11 showed no antibacterial activity in full strength medium (data not shown). In fact, similarly to what was observed with other antimicrobial peptides [40–42], the high ionic strength of MHB could possibly inhibit the interaction between the positive charges of hLF1–11 and the negatively charged bacterial surface. MIC values of each antibiotic were also assessed for comparison in undiluted MHB (Online supplementary materials, Table S1).

The ranges of concentration of the six antimicrobials tested were as follows: 0.125–32 μg/ml for rifampicin, 0.25–256 μg/ml for clarithromycin, 0.125–64 μg/ml for clindamycin, 0.015–16 μg/ml for gentamicin, and 0.06–16 μg/ml for tigecycline. The range of concentrations of hLF1–11 peptide was 2.7–88 μg/ml.

Briefly, the two-fold dilutions of each agent were set up in 100 μl of 1/8 strength Mueller–Hinton broth, and then an equal volume of the mid-log phase bacterial suspension in NaPB was inoculated into each well of the plate at a final concentration of approximately 5×10^5 CFU/ml. Sterility control wells, containing the medium alone, were included in each plate.

After 18–24 h incubation at 37 °C, the MIC of both the peptide and antibiotics were defined on the basis of the turbidity of the wells as the lowest concentration of the agent that produced the complete inhibition of visible growth. A variability of one dilution was considered acceptable to determine the MIC of hLF1–11 and antibiotics for each strain. The fractional inhibitory concentration (FIC) index for the combinations was calculated using the following formula: FIC index = (MIC drug A in combination)/(MIC drug A alone) + (MIC drug B in combination)/(MIC drug B alone). The FIC indices were interpreted as follows: ≤0.5, synergy, >0.5 to ≤4, indifference, and >4, antagonism [43]. FIC index was reported in this study as the mean of the lowest FIC indices of at least three independent experiments.

In addition, for antimicrobial combinations resulting in indifference by the checkerboard method, bactericidal kinetics synergy studies were performed at sub-inhibitory concentrations of each antimicrobial agent (1/2 MIC of hLF1–11, and 1/4 MIC of tigecycline), diluted in 1:16 MHB immediately before use. At 0, 1, 2, 3, 6, and 24 h, serial dilutions of cultures were plated onto solid media to determine the number of CFU per milliliter. Synergy was defined as a decrease in CFU/ml of ≥2 Log of the combination of hLF1–11 and drug, in comparison with its most active constituent [44]. All tests were performed in triplicate.

Hemolysis assay

A hemolysis assay was used to evaluate the cytotoxicity of the peptide and antibiotics alone and in combination. Briefly, blood from three healthy individuals was collected in vacuum tubes containing citrate (Becton Dickinson & Co, BD; Milan, Italy) as anticoagulant. Red blood cells (RBCs) were harvested by centrifugation at 1,600 × g for 5 min at room temperature, washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS to a concentration of 8% (*v*/v). An aliquot (100 μl) of this suspension was transferred into each well of a 96-well microtiter plate and mixed with 100 μl of peptide or antibiotic solution at twice the desired concentration or 50 μl of 4× the peptide and antibiotic for synergy combinations.

After incubation for 1 h at 37 °C, the microtiter plate was centrifuged (1,600 × g, 5 min) and 100 μl of the supernatants were transferred to a flat-bottom 96-well plate for measurement of the hemoglobin release by reading the absorbance at 450 nm. Data were normalized between the 0% hemolysis of RBCs in PBS and 100% hemolysis of RBCs in 1% Triton X-100. The percentage of hemolysis was calculated by the following formula: $(A_{\text{peptide/antibiotic}} - A_{\text{PBS}})/(A_{\text{Triton X-100}} - A_{\text{PBS}}) \times 100\%$.

Statistical analysis

Data were expressed as means ± standard deviation of the mean (SD). Results obtained by the antimicrobial peptide susceptibility test and bactericidal kinetics synergy studies were analyzed by one-way ANOVA test after logarithmic transformation, using GraphPad Instat software (version 6.05 for Windows, La Jolla, CA, USA). Comparisons between the means of the untreated control and treated groups were made applying the Bonferroni post-hoc test. Comparisons between three strains for each peptide concentration were made applying the Tukey post-hoc test. The level of significance was set at a P value of <0.05.

Results

Antimicrobial peptide susceptibility assay

The antimicrobial activity of hLF1–11 was evaluated against three *K. pneumoniae* strains isolated from positive blood cultures. One strain was susceptible to all the tested antimicrobials with the exception of levofloxacin and ciprofloxacin, in addition to ampicillin to which *K. pneumoniae* is naturally resistant. Another strain, further referred to as 1R, showed a multidrug-resistant profile (including colistin resistance), and a third strain had a multidrug-resistant profile but was susceptible to colistin. The complete antimicrobial susceptibility profile of these *K. pneumoniae* strains is reported in Table S2 (Online supplementary materials). The characterization of the MDR strains by the combined disk method revealed that both *K. pneumoniae* strains were KPC-producing. Genotypic characterization by Hy-KPC real-time PCR indicated the presence of bla_{KPC} gene in both the MDR *K. pneumoniae* strains. For the colistin-resistant 1R strain, which was further investigated in synergy studies, the variant of bla_{KPC} gene was evaluated by sequencing, and the results revealed the presence of the variant type 3 of the bla_{KPC} gene (Table 1).

In-vitro killing assays revealed that the hLF1–11-induced bactericidal activity was significantly ($P < 0.05$) higher against the non-carbapenemase-producing than both carbapenemase-producing *K. pneumoniae* strains (colistin-sensitive and colistin-resistant) at all the concentrations tested of hLF1–11 ≥ 88 μM, as shown in Fig. 1.

Synergistic effect of hLF1–11 with antibiotics

Synergy studies combining various concentrations of hLF1–11 with antibiotics were performed by the checkerboard method against the 1R *K. pneumoniae* strain and other strains harboring different bla_{KPC} genes and producing different types of carbapenemases. The MIC values of hLF1–11 and the various antibiotics tested are reported in Table 1. All *K. pneumoniae* strains were inhibited by hLF1–11, with MIC values ranging from 22 to 88 μg/ml.

The results obtained by the combination of hLF1–11 with the various antibiotics, expressed as FIC index, are shown in Table 2. The results revealed that hLF1–11 showed synergism with all antibiotics against at least some *K. pneumoniae* strains. A synergistic effect was observed with rifampicin, clarithromycin, and clindamycin (hydrophobic antibiotics) against all tested strains. The FIC index ranged from 0.22 to 0.47 in the combination of hLF1–11 with rifampicin, from 0.15 to 0.5 and from 0.19 to 0.5 in the combinations with

Table 1 MIC values of the hLF1–11 peptide or antibiotics against *K. pneumoniae* strains used in this study

Strain	MIC (μg/ml)[a]						
	Gene type	hLF1–11	RIF	CLR	CLI	GEN	TGC
K. pneumoniae 1R (colistin-resistant)	$bla_{KPC\text{-}3}$	22–44	16–32	256	32	0.25	0.5–1
K. pneumoniae ATCC® BAA-1705™	$bla_{KPC\text{-}2}$	22–44	16	256	32	4	1
K. pneumoniae	$bla_{OXA\text{-}48}$	44–88	8	128	32	4	1
K. pneumoniae	$bla_{KPC\text{-}2}$	22–44	8–16	256	32	8	1
K. pneumoniae (colistin-susceptible)	$bla_{KPC\text{-}3}$	22–44	8	128	32	0.125–0.25	0.5
K. pneumoniae	$bla_{VIM\text{-}1}$	44–88	8	128	16	0.125	0.5
K. pneumoniae (susceptible isolate)	None	22	ND	ND	ND	ND	ND
K. pneumoniae (colistin-susceptible)	bla_{KPC}	44	ND	ND	ND	ND	ND

RIF rifampicin, *CLR* clarithromycin, *CLI* clindamycin, *GEN* gentamicin, *TGC* tigecycline

[a] MIC values were obtained by microdilution method in 1:16 diluted MHB

ND not determined

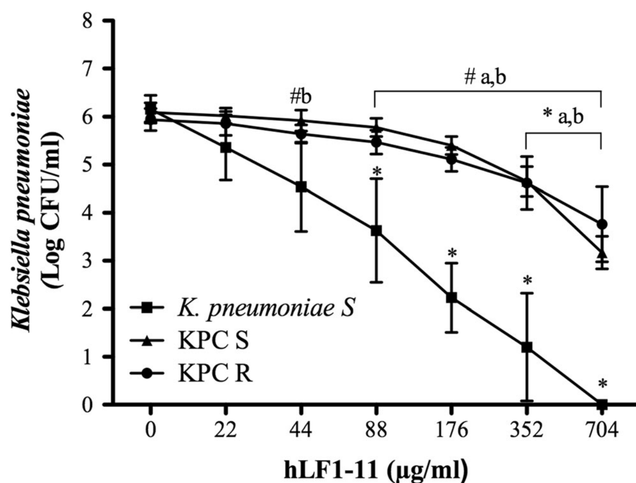

Fig. 1 Antibacterial activity of hLF1–11 at different concentrations against three *K. pneumoniae* strains: a clinical susceptible *K. pneumoniae* strain (*S*, *square*), a colistin-resistant KPC *K. pneumoniae* strain (*KPC R*, *circle*, *a*), and a colistin-sensitive KPC *K. pneumoniae* strain (*KPC S*, *triangle*, *b*). Data are means ± SD of at least three independent experiments. *K. pneumoniae* cells (10^6 CFU/ml) were incubated with hLF1–11 for 1 h at 37 °C. The number of viable bacterial cells was determined by plating serial dilutions of each sample onto blood agar plates. * Significantly different ($P < 0.05$, ANOVA, Bonferroni test) as compared to untreated *K. pneumoniae* cells; # Significantly different ($P < 0.05$, ANOVA, Tukey test) from values obtained with the susceptible *K. pneumoniae* strain

clarithromycin and clindamycin respectively. In the presence of hLF1–11, the MIC of rifampicin was reduced by 64-fold (from 8 μg/ml to 0.125 μg/ml) for OXA-48 and VIM-1 *K. pneumoniae* strains, by 32-fold (from 16 μg/ml to 0.5 μg/ml and from 8 μg/ml to 0.25 μg/ml, respectively) for *K. pneumoniae* ATCC® BAA-1705™ and KPC-3 (colistin-sensitive), and by 8- to 16-fold for KPC 1R and KPC-2 strains.

In the peptide–clarithromycin combination, the MIC of the antibiotic was decreased from 4- to 64-fold, with the highest reductions observed against KPC-2 (from 32- to 64-fold), ATCC® BAA-1705™, and VIM-1 (both from 16- to 32-fold) strains. Among all strains, KPC 1R and KPC-3 showed the highest values of FIC indices.

In the peptide–clindamycin combination, the MIC of the antibiotic was decreased from 4- to 32-fold, with the highest reductions observed against VIM-1 (from 8- to 32-fold), and ATCC® BAA-1705™ (from 8- to 16-fold) strains. Among all strains, KPC 1R and KPC-3 showed the highest values of FIC indices.

Synergy was also obtained when hLF1–11 was tested in combination with gentamicin, as evidenced by FIC indices ranging from 0.21 to 0.5, and with tigecycline with FIC indices of 0.38–0.5. In contrast, no synergistic effect was observed with the hLF1–11 peptide-tigecycline combination against the KPC 1R strain and *K. pneumoniae* harboring the bla_{KPC-3} gene, with FIC indices of 0.84 and 0.75 respectively. Antibacterial kinetics experiments also showed no synergism in the peptide–tigecycline combination against the 1R strain at 24 h (Fig. 2), but revealed a synergistic effect at earlier time points. In fact, as shown in Fig. 2, the results of antibacterial kinetics experiments revealed a 3 Log CFU reduction at 6 h by the peptide–antibiotic combination in comparison to its most active constituent [44].

Hemolysis assay

A hemolysis assay was performed to evaluate possible toxic effects of the hLF1–11 peptide and antibiotics on human red

Table 2 Effect of the combination of the hLF1-11 peptide and antibiotics, expressed as FIC index, against carbapenemase-producing *K. pneumoniae* strains harboring different resistance genes

		Lowest FIC index[a]				
Strain	Gene type	RIF	CLR	CLI	GEN	TGC
K. pneumoniae 1R (colistin-resistant)	bla_{KPC-3}	0.35 (2/11)	0.5 (64/11)	0.5 (8/11)	0.31 (0.03/5.5; 0.06/2.7)	0.84 (0.125/22; 0.25/22; 0.5/11)
K. pneumoniae ATCC® BAA-1705™	bla_{KPC-2}	0.23 (0.5/5.5; 4/2.7)	0.18 (8/5.5; 16/5.5)	0.33 (2/5.5; 4/5.5)	0.38 (0.5/5.5; 1/2.7)	0.38 (0.25/5.5; 0.125/11)
K. pneumoniae	bla_{OXA-48}	0.22 (0.125/11)	0.19 (8/5.5; 16/5.5; 16/2.7)	0.35 (2/11; 4/11)	0.38 (0.5/11)	0.5 (0.25/11)
K. pneumoniae	bla_{KPC-2}	0.35 (0.5/5.5; 1/5.5)	0.15 (4/5.5; 8/5.5)	0.42 (8/2.7; 8/5.5; 4/5.5)	0.5 (2/5.5)	0.5 (0.25/5.5)
K. pneumoniae (colistin-susceptible)	bla_{KPC-3}	0.47 (0.25/11; 0.5/11)	0.46 (16/5.5; 32/5.5)	0.5 (8/5.5)	0.23 (0.015/5.5; 0.03/2.7)	0.75 (0.25/11; 0.06/22)
K. pneumoniae	bla_{VIM-1}	0.27 (0.125/11)	0.18 (4/11; 8/11)	0.19 (0.5/11; 2/5.5)	0.21 (0.015/2.7; 0.015/5.5)	0.38 (0.06/22; 0.125/11)

RIF rifampicin, *CLR* clarithromycin, *CLI* clindamycin, *GEN* gentamicin, *TGC* tigecycline

[a] Mean of the lowest FIC indices of at least three independent experiments. Mean FIC index values ≤ 0.5 indicate synergism. The numbers in parentheses are the MICs (μg/ml) of the antibiotic (first number, before the slash) and of hLF1-11 (second number, after the slash) in the combinations. When more than one effective antibacterial combination was observed, all effective combinations have been reported separated by semicolon

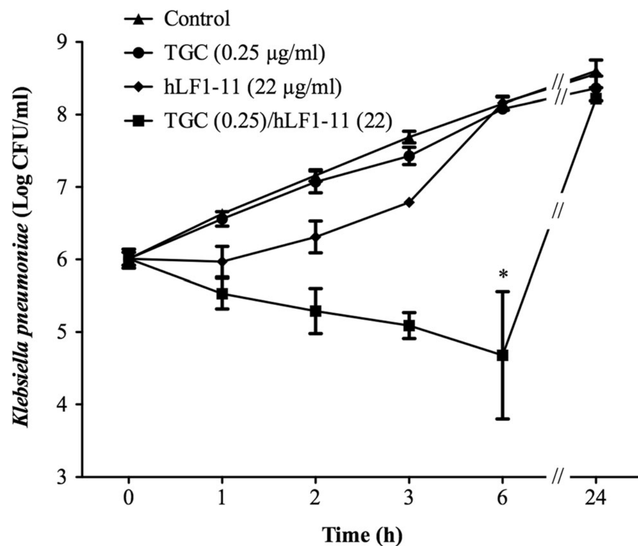

Fig. 2 Kinetics of the antibacterial activity of hLF1–11 and tigecycline against KPC 1R *K. pneumoniae* strain. *K. pneumoniae* cells (10^6 CFU/ml) were incubated with 22 μg/ml hLF1–11 (*diamond*), 0.25 μg/ml tigecycline (*circle*), the combination of the same concentrations of hLF1–11 and tigecycline (*square*), or no treatment (*triangle*). The number of viable bacterial cells was determined at 0, 1, 2, 3, 6, and 24 h by plating serial dilutions of each sample onto blood agar plates. Data are means ± SD of at least three independent experiments. * Synergistic effect of the combination of hLF1–11 and tigecycline in comparison to its most active constituent ($P < 0.05$, ANOVA, Bonferroni test)

blood cells (Fig. 3). The results revealed that hLF1–11 alone had no hemolytic activity (<1%) even at 10× the highest MIC value (880 μg/ml). In addition, all the tested antibiotics alone showed no hemolysis (≤1%), with the exception of rifampicin, which exhibited 1–5% hemolysis at 8–32 μg/ml and 37% hemolysis at 10× the highest MIC value (320 μg/ml). All combinations of the peptide with antibiotics showed no hemolysis (<1%) even at 10× MIC for both constituents (Fig. 4), with the exception of the combination of hLF1–11 with rifampicin, which exhibited no hemolysis at MIC (<1%) but 3% hemolysis at 10× MIC.

Discussion

Carbapenem-resistant *K. pneumoniae* strains are often resistant to multiple classes of antibiotics, including fluoroquinolones, aminoglycosides, and β-lactams [45] and, therefore, treatment of infections caused by such strains often meets with failure, and is associated with high mortality rates. Combination therapies have been shown to significantly improve the therapeutic efficacy in the treatment of bacterial infections [46], and there is evidence suggesting that emergence of antimicrobial resistance may be reduced by using combination therapy regimens [47, 48]. However, this is a controversial issue and some studies indicate that, unless highly inhibitory antibiotic doses are maintained until the pathogen is eradicated, combination therapy can have the opposite effect, i.e., to accelerate the emergence and spread of MDR bacterial strains [49, 50]. Another, recently proposed approach to combat infections caused by MDR bacterial strains consists in combining antibiotics and antimicrobial peptides [51]. These latter show broad-spectrum antimicrobial activity, frequently show strong synergism with conventional antibiotics [52], and have been suggested to help in preventing or delaying the emergence of antibiotic resistance [51, 52].

The present study aimed at evaluating the antibacterial activity of hLF1–11, alone or in combination with various antimicrobial drugs, against different strains of *K. pneumoniae*. The mechanism of action of cationic AMP is commonly

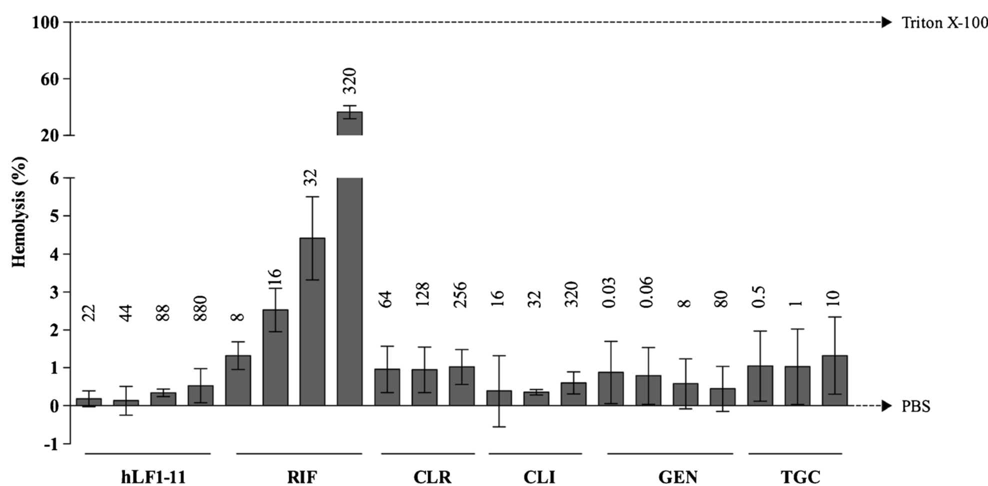

Fig. 3 Hemolytic activity of peptide and antibiotics alone at various MICs. The *values above the bars* indicate the tested concentrations (μg/ml). The antibiotic or the peptide was incubated with 8% RBC suspension. The results are expressed as mean of percentage of hemolysis. RBCs incubated with 1% Triton X-100 and PBS (untreated) were considered as 100% and 0% hemolysis respectively. The percentage of hemolysis was calculated as follows: $(A_{peptide/antibiotic} - A_{PBS})/(A_{Triton\ X\text{-}100} - A_{PBS}) \times 100\% \pm$ SD from three independent experiments. *RIF*, rifampicin; *CLR*, clarithromycin; *CLI*, clindamycin; *GEN*, gentamicin; *TGC*, tigecycline

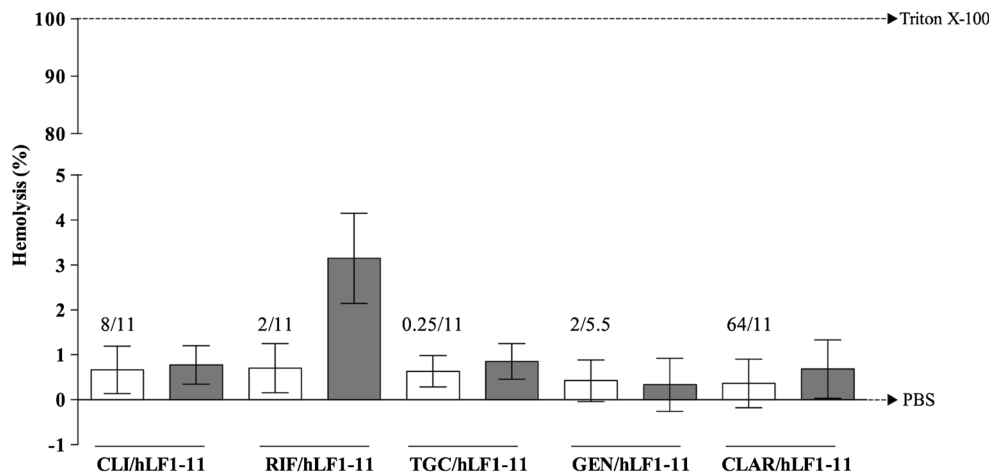

Fig. 4 Hemolytic activity of the combination of the hLF1–11 peptide and antibiotics at MICs (*open bars*) and 10× MICs (*gray bars*). The *values above the open bars* indicate the tested concentrations (μg/ml). The tested concentrations are reported as x/y, where x and y represent the concentrations of the antibiotic and peptide respectively. The antibiotic and peptide were incubated with 8% RBC suspension. The results are expressed as mean of percentage of hemolysis. RBCs incubated with 1% Triton X-100 and PBS (untreated) were considered as 100% and 0% hemolysis respectively. The percentage of hemolysis was calculated as follows: $(A_{peptide/antibiotic} - A_{PBS})/(A_{Triton\ X\text{-}100} - A_{PBS}) \times 100\% \pm SD$ from three independent experiments. *RIF*, rifampicin; *CLR*, clarithromycin; *CLI*, clindamycin; *GEN*, gentamicin; *TGC*, tigecycline

related to their ability to interact with negatively charged components of cell membranes, and a correlation between colistin resistance and cross-resistance to host antimicrobial peptides has been reported, thus suggesting the existence of shared mechanisms of action for these compounds [53]. Therefore, the hLF1–11-induced bactericidal activity was evaluated against two MDR *K. pneumoniae* strains differing for colistin susceptibility and a *K. pneumoniae* strain susceptible to most of the antibiotics tested. The results revealed that the hLF1–11-induced bactericidal activity was significantly ($P < 0.05$) higher against a non-carbapenemase-producing *K. pneumoniae* than a colistin-sensitive and a colistin-resistant carbapenemase-producing *K. pneumoniae* strain in an in-vitro killing assay. This evidence indicates that resistance to colistin and to the hLF1–11 peptide are not strictly associated. Therefore, other distinct mechanisms of resistance to the hLF1–11 peptide and to colistin should also be considered [54].

The main conclusion from the present data is that hLF1–11 is a promising candidate for combination therapies with various antibiotics in the treatment of infections caused by MDR *K. pneumoniae* strains. This conclusion is based on the following findings. First, the hLF1–11 peptide exerted synergistic effects in combination with most of the antibiotics tested by the checkerboard assay. Rifampicin, clarithromycin, or clindamycin, which are hydrophobic antimicrobial drugs, are normally not able to permeate through the outer membrane of Gram-negative bacteria, thereby being ineffective against these microorganisms [55]. Indeed, hLF1–11 induced up to a 64-fold reduction in the MIC of these hydrophobic antibiotics and a 4- to 16-fold reduction in the MIC of gentamicin or tigecycline against the tested *K. pneumoniae* strains. These results suggest a hLF1–11-induced sensitizing effect on KPC *K. pneumoniae* strains to antibiotics. The mechanism underlying the synergistic effect between hLF1–11 and otherwise impermeable hydrophobic antibiotics might be the result of a transient loss of membrane potential induced by hLF1–11 and subsequent increase in cell membrane permeabilization. Further studies will help to elucidate the mechanism of action underlying these synergistic effects. Second, the highest FIC indices by the checkerboard assay, indicating indifference, were observed in the combinations of hLF1–11 and tigecycline against the two *K. pneumoniae* strains harboring the $bla_{KPC\text{-}3}$ gene. However, antibacterial kinetics studies, performed against *K. pneumoniae* 1R showed a synergistic effect exerted by the combination of hLF1–11 and tigecycline at 6 h. In agreement with the results obtained by the checkerboard assay, no synergistic effect was observed at 24 h. Due to the chemical nature of the peptide, it is possible that hLF1–11 was progressively inactivated by bacterial cell components, such as proteases, released by dead cells after incubation with the antimicrobial peptide. A multiple daily administration regimen for the combination hLF1–11/tigecycline as well as for other antimicrobial peptide/antibiotic combinations might be considered in order to achieve complete eradication of bacteria. Chemical modification of the peptide enhancing its stability in biological fluids might also be attempted [56]. Alternatively, resumption of bacterial growth might be due to rapid evolutionary adaptation of *K. pneumoniae* persistence, though this phenomenon is usually associated with repeated antibiotic application rather than with a single-dose exposure [57]. Further studies will be needed to shed light on this issue. Third, all combinations of the peptide with antibiotics showed no hemolytic activity (<1%) even at 10× MIC, with the only exception of rifampicin, which exhibited 3% hemolysis at 10× MIC in combination with hLF1–11. The

latter observation is not surprising, since the rifampicin-induced hemolytic activity by eryptosis has already been described [58]. These results indicate that hLF1–11 might be safe to be used in combination with antibiotics in the treatment of infections caused by KPC *K. pneumoniae* strains.

Overall, the results of the present study indicate that a combination therapy consisting of hLF1–11 and conventional antibiotics may be considered, and might be helpful as a last resort to treat infections sustained by antibiotic resistant bacterial strains. Such combination therapies represent a promising approach to treat infections caused by MDR *K. pneumoniae* strains for which conventional antibiotics are no longer effective and hydrophobic antibiotics are not indicated. Since the ionic strength in biological fluids may affect the antibacterial activity of hLF1–11, further studies will be needed to assess the in-vivo efficacy of hLF1–11, alone or in combination with antibiotics, against MDR *K. pneumoniae* strains, and to elucidate the mechanisms of action underlying the sensitizing effect of hLF1–11 to antibiotics.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was notified to 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. These samples were anonymized by the clinical personnel. Research personnel received and used these samples anonymously. For this type of study, no written informed consent was necessary.

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

Informed consent For this type of study, formal consent is not required.

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