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ORIGINAL RESEARCH

Biofilm infections between Scylla and Charybdis: interplay of host antimicrobial peptides and antibiotics

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Purpose: The aim of this study is to improve the anti-biofilm activity of antibiotics. We hypothesized that the antimicrobial peptide (AMP) complex of the host's immune system can be used for this purpose and examined the assumption on model biofilms.

Methods: FLIP7, the AMP complex of the blowfly *Calliphora vicina* containing a combination of defensins, cecropins, diptericins and proline-rich peptides was isolated from the hemolymph of bacteria-challenged maggots. The complex interaction with antibiotics of various classes was studied in biofilm and planktonic cultures of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* by the checkerboard method using trimethyl tetrazolium chloride cell viability and crystal violet biofilm eradication assays supplemented with microscopic analysis.

Results: We found that FLIP7 demonstrated: high synergy (fractional inhibitory concentration index <0.25) with meropenem, amikacin, kanamycin, ampicillin, vancomycin and cefotaxime; synergy with clindamycin, erythromycin and chloramphenicol; additive interaction with oxacillin, tetracycline, ciprofloxacin and gentamicin; and no interaction with polymyxin B. The interaction in planktonic cell models was significantly weaker than in biofilms of the same strains. The analysis of the dose–effect curves pointed to persister cells as a likely target of FLIP7 synergistic effect. The biofilm eradication assay showed that the effect also caused total destruction of *S. aureus* and *E. coli* biofilm materials. The effect allowed reducing the effective anti-biofilm concentration of the antibiotic to a level well below the one clinically achievable (2–3 orders of magnitude in the case of meropenem, ampicillin, cefotaxime and oxacillin).

Conclusion: FLIP7 is a highly efficient host antimicrobial system helping antibiotics to overcome biofilm barriers through persisters' sensitization and biofilm material destruction. It is promising for the treatment of biofilm infections as an adjuvant of various small-molecule antibiotics.

Keywords: insect antimicrobial peptides, antibiotics, synergy, biofilms, persisters, *Calliphora vicina*

Introduction

Most bacteria form biofilms in order to survive in the host organism. These biofilms are sedimented multicellular communities embedded in a biopolymer matrix. Biofilms account for over 80% of microbial infections that cause nonhealing wounds, ulcers and lesions of the skin, mucosa and internal organs.[1] A growing body of data also points to the important role of biofilms in the occurrence of diseases that are usually not considered to be infectious, such as cancer[2–4] and cardiovascular disorders.[5,6] Moreover, biofilms, which invade the surfaces of catheters, implants and other medical devices,

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make a significant contribution to morbidity and economic losses from bacterial infections.[7] The transition of planktonic bacteria to biofilms gives them multifactorial protection against the host's immune system and allows persistence in the body for an unlimited time.[8,9] Enhanced resistance to antibiotics and antiseptics is another clinically important factor in biofilm infections.[10–14] The escalating prevalence of antibiotic-resistant strains makes the task of treating biofilms even more difficult. Thus, the search for new ways of treating biofilm infections is one of the important areas of health care progress.

Over the last three decades, animal antimicrobial peptides (AMPs) have attracted attention as a promising platform for the development of new antibacterial drugs. More than 2600 sequences of AMPs toxic to planktonic bacteria have been identified in animals, plants, fungi and microorganisms.[15] Much less is known about anti-biofilm AMPs, even though some of them exhibit strong cell-killing and/or matrix-destroying activity against bacterial biofilms.[16–19] Using the checkerboard method, it was possible to detect two-component AMP–AMP[20,21] and AMP–antibiotic[17,19,22] combinations with a synergistic anti-biofilm effect. However, further progress in the development of such combinations encounters significant technical difficulties, since not only the type of interaction (synergism, additivity, antagonism) but also other parameters like antibacterial activity spectrum, toxicity, the rate of resistance development and so on should be estimated. The search for combinations comprising three or more components seems to be an almost insoluble problem from this point of view.

At the same time, the immune systems of multicellular animals exploit multicomponent AMP systems optimized by natural selection in all the parameters important for the survival of the host organism. Although the idea of using natural AMP complexes as a platform for the development of antimicrobial drugs seems attractive from various points of view, it has so far been neglected. Only recently, the key benefits of this approach have received experimental confirmation. Particularly, experimental evolution studies revealed that none of the Gram-negative bacteria which were tested were able to acquire resistance to the host AMP complexes of blowfly *Calliphora vicina* and other Calliphoridae in contrast to conventional antibiotics.[23] A further study has shown that a *C. vicina* complex containing a combination of four AMP families (defensins, cecropins, diptericins and proline-rich peptides) is active against not only planktonic bacteria but also their biofilms.[24] Thus, the natural AMP complex is superior to conventional antibiotics in at least two respects: the ability to prevent the development of acquired resistance and the effectiveness against biofilms. The question arises as to whether it is possible to combine the merits of host AMP complexes and antibiotics and thus create a more effective approach to the treatment of biofilm infections.

In this study, we tried to find an answer to this question by investigating the interaction of a *C. vicina* AMP complex referred to as FLIP7 (Fly Larvae Immune Peptides) and antibiotics of the main classes using the checkerboard method and in vitro models of biofilms formed by clinically relevant pathogens *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Materials and methods

C. vicina AMP complex

The sample of FLIP7 was isolated from the hemolymph of blue blowfly *C. vicina* diapausing maggots. The maggots were inoculated with *E. coli* M17 strain (Microgen, Moscow, Russia) to induce an immune response and left overnight at 25°C. The hemolymph was collected in ice-cold tubes through a cuticle puncture, frozen and kept at −70°C until use. The thawed hemolymph was acidified with 0.1% trifluoroacetic acid (TFA) to a final concentration of 0.05%, and the insoluble particles were removed by centrifugation (30 minutes at 8000 × *g* at 4°C). The supernatant was applied to reversed-phase Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) stabilized by 0.05% TFA in the amount of 5 mL/g of sorbent. Highly hydrophilic compounds were removed by cartridge washing with 0.05% TFA. The compounds absorbed in the cartridge were eluted with 50% acetonitrile solution acidified with 0.05% TFA, lyophilized (FreeZone; Labconco, Kansas City, MO, USA) and stored at −70°C. Prior to use, the lyophilized sample was dissolved in deionized water (50 mg/mL), sterilized by filtration through a membrane with a pore size of 0.22 μm (Milliex-GS; Merck Millipore, Billerica, MA, USA) and frozen at −70°C.

The sample used in this work was structurally characterized in our previous paper using a combination of liquid chromatography/mass spectrometry and transcriptome methods.[24] Ten peptides belonging to four AMP families (defensins, diptericins, cecropins and proline-rich peptides) of the insects were deciphered, although some additional AMPs found in the complex remained uncharacterized. The database of mRNAs isolated from bacteria-challenged *C. vicina* maggots and available at https://www.ncbi.nlm.nih.gov/sra/SRX2523753 (submission number SRR5210297)

predicts the existence of additional AMP isoforms especially diverse in the family of diptericins.

Antibiotics

The following commercial preparations were used in the experiments: aminoglycosides amikacin sulfate (Sintez, Kurgan, Russia), kanamycin sulfate (Sintez) and gentamicin sulfate (AppliChem, Darmstadt, Germany), natural penicillin oxacillin sodium (Sintez), aminopenicillin ampicillin sodium (Sintez), carbopenem meropenem trihydrate (AstraZeneca, Cambridge, UK), third-generation cephalosporin cefotaxime sodium (Abolmed, Moscow, Russia), naturally occurring polypeptide polymyxin B sulfate (AppliChem), glycopeptide vancomycin (Kraspharma, Krasnoyarsk, Russia), lincosamid clindamycin phosphate (Hemopharm, Vršac, Serbia), macrolide erythromycin phosphate (Sintez), second-generation fluoroquinolone ciprofloxacin hydrochloride (AppliChem), chloramphenicol sodium succinate (AppliChem), tetracycline hydrochloride (AppliChem) and antiseptic benzalkonium chloride (Fluka, Buchs, Switzerland). The antibiotics were dissolved in sterile deionized water at a concentration of 10, 1 and 0.1 mg/mL, aliquoted in 0.05 mL volumes and kept at −70°C until use. Chloramphenicol and tetracycline were dissolved in 50% ethanol in sterile deionized water.

Bacteria

E. coli ATCC 25922 and *P. aeruginosa* ATCC 27583 strains as well as clinical strains of *S. aureus* 203, *K. pneumoniae* 145 and *A. baumannii* 28 were used as model biofilm-forming bacteria. The annotated genome assembly of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27583 is available at the links http://www.ncbi.nlm.nih.gov/nuccore/CP009072 and http://www.pseudomonas.com/strain/show/3104, respectively. The *A. baumannii* 28 genome is available at https://www.ncbi.nlm.nih.gov/nuccore/NZ_MAFT00000000. Strain genome sequencing predicts resistance to aminoglycosides, beta-lactams and chloramphenicol. The clinical strain of *K. pneumoniae* 145 originates from the surgery clinic of the Kirov Military Medical Academy (St. Petersburg, Russia), and the strains of *A. baumannii* 28 and *S. aureus* 203 originate from the collection of The Northwestern State Medical Mechnikov University and Institute of Experimental Medicine (St. Petersburg, Russia), respectively. The profiles of the clinical strains' antibiotic resistance were determined as recommended.[47] The strains were classified as susceptible, intermediate or resistant to the antibiotic in testing with disc diffusion method. To test the resistance profiles of *K. pneumoniae* 145, the following antibiotics were used: amikacin, amoxicillin/clavulanic acid, gentamicin, imipenem, meropenem, cefepime, cefoperazone, cefoperazone/sulbactam, cefotaxime, ceftazidime, ciprofloxacin, piperacillin/tazobactam and tigecycline (Oxoid Limited, Basingstoke, UK). The strain was found to be resistant to all the antibiotics that were tested except imipenem, meropenem and tigecycline. The *S. aureus* 203 strain was found to be sensitive to all the antibiotics tested.

Biofilm formation

The preparation of 24-hour-old biofilms corresponded to the previously described procedure ensuring the formation of a dense biofilm by the strains used in experiments.[24] The biofilm formation was confirmed by the staining of 24-hour biofilm with crystal violet dye. The bacteria were cultured in 5 mL of LB liquid nutrient medium (Thermo Fisher Scientific, Waltham, MA, USA) for 18–20 hours at 37°C. Overnight cultures were adjusted to 5×10^5 CFU/mL measured by optical density (OD). One-hundred-microliter aliquots of the diluted bacterial suspension were inoculated into each well of a 96-well flat-bottomed polystyrene plate (Sarstedt AG & Co., Nümbrecht, Germany) and incubated in a humidified incubator for 24 hours at 37°C. The negative control was an LB liquid nutrient medium.

TTC cell-killing assay

The effect of antimicrobial agents on the viability of biofilm bacteria was assessed using a standard trimethyl tetrazolium chloride (TTC) test identical to that described previously.[24] The 24-hour biofilms, formed in a 96-well tissue culture microtiter plate, were washed three times with 200 µL of sterile physiological buffered saline (PBS) to remove unattached bacteria and then air-dried. An antimicrobial agent dissolved in 100 µL LB liquid nutrient medium (Thermo Fisher Scientific) was added to each corresponding well, and the plates were incubated for 24 hours at 37°C in a humidified incubator. Then, 11 µL of 0.2% TTC (Lenreactiv, St. Petersburg, Russia) was added to a final concentration of 0.02%. After 1 hour of incubation at 37°C, the OD_{540} was measured on the Epoch microplate reader (BioTek Instruments, Winooski, VT, USA). The mean OD_{540} value of 48-hour biofilm cells without antimicrobial agent treatment was set as the control. Each experiment was performed in two independent assays. The minimum biofilm-inhibiting concentration (MBIC) was assessed with $MBIC_{90}$, the concentration decreasing TTC staining by 90% compared to the control.

Crystal violet biofilm eradication assay

A standard crystal violet biofilm eradication assay was performed with minor modifications as described previously.[24]

This method gives the total amount of the biofilm material binding to the dye, including extracellular and cellular components. The 24-hour biofilms, formed in a 96-well tissue culture microtiter plate, were washed three times with 200 μL of sterile PBS solution and air-dried. One hundred microliters of antimicrobial composition dissolved in LB liquid nutrient medium (Invitrogen, Carlsbad, CA, USA) was added to each corresponding well, and the plates were incubated for 24 hours at 37°C. Then, the waste media was removed, and the plates were washed three times with 200 μL PBS solution, air-dried and stained with crystal violet 0.1% (in water) (Lenreactiv, St. Petersburg, Russia) for exactly 2 minutes. The stained biofilms were washed three times with 200 μL PBS solution, air-dried and solubilized with 200 μL of 95% ethanol for 1 hour. Then, the biofilm-associated dye was measured at OD_{570} using the Epoch reader (BioTek Instruments). Each experiment was performed in two independent assays. The minimum biofilm eradication concentration (MBEC) was assessed with $MBEC_{90}$, which is the concentration of antimicrobials decreasing crystal violet binding in preformed biofilms by 90% compared to untreated control.

Planktonic cells' viability assessment

The standard microdilution method was carried out for the determination of planktonic cells minimum inhibiting concentration (MIC), as recommended.[48] The initial inoculum was grown on a solid LB agar nutritive medium (Invitrogen, Carlsbad, CA, USA). Individual colonies were collected, transferred into liquid medium (Luria broth base, 25 g/L) and incubated overnight at 37°C. Individual wells of a 96-well tissue culture plate (Sarstedt, Newton, NC, USA) containing 100 μL of LB liquid nutrient medium (Invitrogen, Carlsbad, CA, USA) with doubling antimicrobial dilutions were inoculated with approximately 5×10^5 CFU/mL of test bacteria. Microtiter plates were incubated for 20 hours at 37°C. The cells' viability was determined with the TTC method as described above. The MIC was assessed with MIC_{90}, the concentration decreasing TTC staining by 90% compared to the untreated control.

The AMP complex and antibiotics interaction studies

To establish the type and quantitative characteristics of the interaction of *C. vicina* AMP complex and antibiotics of different classes, the checkerboard titration method with some modifications was used.[25] The 24-hour biofilms formed in a 96-well tissue culture microtiter plate were washed three times with 200 μL of sterile PBS and air-dried. In another 96-well microplate, combinations of FLIP7 and antibiotics were prepared in such a way that two-fold dilutions of FLIP7 were placed in horizontal rows of wells, and two-fold dilutions of antibiotics were placed in vertical rows. Then, 100 μL of the contents of each well from this microplate was transferred to a biofilm microplate and incubated for 24 hours at 37°C. The biofilm state was evaluated by the TTC and crystal violet methods as described above. All the experiments were performed in two independent replicates. In experiments with planktonic cultures, dilutions of FLIP7 and an antibiotic were pooled at 25 μL in wells of the microplate according to the scheme, and 50 μL of bacterial suspension with a cell concentration of 10^6 CFU/mL was added to each well. The microplate was incubated for 24 hours at 37°C, and then the contents of the wells were stained with TTC as described above.

The effects of the combinations were examined by calculating the fractional inhibitory concentration index (FICI) of each combination as follows: [(MIC of drug A, tested in combination)/(MIC of drug A, tested alone)] + [(MIC of drug B, tested in combination)/(MIC of drug B, tested alone)]. In the TTC test and the crystal violet test, the values of $MBIC_{90}$ and $MBEC_{90}$ were used instead of MIC. The profile of the combination was defined as synergistic if the FICI was ≤0.5, indifferent if the FICI was >0.5 but ≤4.0 and antagonistic if the FICI was >4. The ratio of the biofilm $MBIC_{90}$ to the planktonic culture MIC_{90} determined in the TTC cell viability assay was used as a quantitative characteristic of the biofilm-related resistance (BRR).

Biofilm microscopic visualization

A coverslip of 24 × 24 mm (Thermo Fisher Scientific Gerhard Menzel B.V. & Co. KG, Braunschweig, Germany) was placed in each well of six-well tissue culture microtiter plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). The wells were filled with 2.8 mL of *S. aureus* 203 suspension, prepared as mentioned in the "Biofilm formation" section, and the plates were incubated for 24 hours at 37°C. Then, the 24-hour biofilms were washed three times with 3 mL of sterile PBS to remove unattached bacteria and filled with fresh culture medium (control) or a medium supplemented with FLIP7, meropenem or a combination of both. The plates were incubated for another 24 hours at 37°C, and then coverslips were removed from the wells, washed in PBS, placed on a glass slide upside down and photographed through a Leica DMI 2500 microscope (Leica Microsystems, Wetzlar, Germany) with Nomarski optics at ×400 and ×1000 magnification.

Statistical methods

Statistical processing of data was performed using the Primer of Biostatistics software, version 4.03. Statistical significance of the differences was analyzed with ANOVA and *Z*-test considering *P*-values <0.05 as significant.

Results

BRR profiles quantification

For the analysis of model biofilms' recalcitrance to antibiotics, the ratio of biofilm $MBIC_{90}$ and planktonic cells MIC_{90} was used as a quantitative characteristic referred here to as BRR. The obtained values of BRR (Table 1) show that bacteria in the state of biofilm have increased recalcitrance to the majority of antibiotics studied compared to planktonic cells of the same strain. However, the level of BRR varies widely, depending on the type of antibiotic and the type of bacteria. The maximum BRR growth was registered in *S. aureus*, particularly to meropenem (over 2000-fold), ampicillin, amikacin and kanamycin. A significant BRR increase was also noted for vancomycin, benzalkonium chloride and erythromycin (10- to 84-fold). Resistance to oxacillin and chloramphenicol only slightly exceeded the level of planktonic cells (1.25- to 2.4-fold).

The *E. coli* biofilm exhibited maximum resistance growth to meropenem (125-fold), and a less significant resistance growth to cefotaxime and polymyxin B (21- to 31-fold), whereas resistance to ciprofloxacin, tetracycline, gentamicin and chloramphenicol was only slightly different from the planktonic cells level.

Meropenem resistance changes were also tested in *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. Biofilms of *P. aeruginosa* and *A. baumannii* demonstrated intermediate 11- to 20-fold resistance growth, whereas in *K. pneumoniae* it was 703-fold.

Table 1 Biofilm-related resistance and FLIP7–antibiotic interaction in biofilms (TTC assay)

Strain/antibiotic	BRR[1] quantification			FLIP7 and antibiotic interplay in biofilms			
	Planktonic cells MIC_{90} (μg/mL)	Biofilm $MBIC_{90}$ (μg/mL)	BRR[1]	FICI	Interaction type	Antibiotic $MBIC_{90}$ in FLIP7 presence (μg/mL)[2]	Fold reduction[3]
Staphylococcus aureus							
Meropenem	0.02 ± 0.02	>50	>2000	0.168	Syn	<0.1	>500
Amikacin	1.88 ± 0.00	>500	>266	0.087	Syn	1.5 ± 0.0	>333
Kanamycin	3.51 ± 1.17	>500	>142	0.107	Syn	3.0 ± 0.0	>168
Ampicillin	0.03 ± 0.01	19.5 ± 3.94	650	<0.208	Syn	<1.0	>24
Vancomycin	0.41 ± 0.19	34.4 ± 15.6[4]	83.9	0.165	Syn	1.5 ± 0.9	22.9
Benzalkonium Cl	0.30 ± 0.00	12.0 ± 0.00	40	0.749	Add	2.25 ± 0.75	5.3
Clindamycin	75.0 ± 0.0	>250	>3	0.355	Syn	10.0 ± 2.0	>25
Erythromycin	0.94 ± 0.00	9.4 ± 0.00	10	0.422	Syn	0.45 ± 0.15	20.9
Oxacillin	0.05 ± 0.00	0.12 ± 0.00	2.4	0.583	Add	0.01 ± 0.003	9.2
Tetracycline	0.12 ± 0.00	0.15 ± 0.00	1.25	<0.749	Add	<0.10	>1.5
Chloramphenicol	4.70 ± 0.00	5.0 ± 0.00	1.1	0.417	Syn	1.0 ± 0.5	5.0
Escherichia coli							
Meropenem	0.03 ± 0.00	3.75 ± 0.00	125	0.297	Syn	<0.02	>188
Cefotaxime	0.09 ± 0.00	2.81 ± 0.94	31.2	0.179	Syn	0.07 ± 0.01	40
Polymyxin B	0.34 ± 0.11	7.03 ± 2.34	20.7	1.042	Ind	9.4 ± 0.0	0.75
Ciprofloxacin	0.03 ± 0.00	0.06 ± 0.00	2	0.542	Add	<0.02	>3
Tetracycline	0.46 ± 0.00	0.9 ± 0.30	1.9	0.708	Add	0.15 ± 0.0	6
Gentamicin	4.70 ± 0.00	6.25 ± 3.13	1.3	0.794	Add	0.6 ± 0.0	10.4
Chloramphenicol	4.69 ± 0.00	3.0 ± 0.00	0.6	0.667	Add	1.50	2
Pseudomonas aeruginosa							
Meropenem	0.94 ± 0.31	10.9 ± 1.6	11.6	0.407	Syn	2.4 ± 0.0	4.5
Klebsiella pneumoniae							
Meropenem	0.01 ± 0.00	7.03 ± 2.34	703	0.583	Add	0.25 ± 0.05	28.1
Acinetobacter baumannii							
Meropenem	0.88 ± 0.13	9.4 ± 0.0	10.7	0.370	Syn	1.2 ± 0.0	7.8

Notes: [1]Biofilm-related resistance determined as antibiotic $MBIC_{90}$ to planktonic cells MIC_{90} ratio. [2]$MBIC_{90}$ of antibiotic in combination with FLIP7 concentration causing maximum fold reduction. [3]Ratio of antibiotic $MBIC_{90}$ in the absence and presence of FLIP7 concentration causing maximum antibiotic fold reduction. [4]The difference from the planktonic culture MIC_{90} was statistically unreliable ($P = 0.161$). Data presented as mean ± SE.

Abbreviations: TTC, trimethyl tetrazolium chloride; BRR, biofilm-related resistance; MIC, minimum inhibiting concentration; MBIC, minimum biofilm-inhibiting concentration; FICI, fractional inhibitory concentration index; Syn, synergy; Add, additivity; Ind, independence.

Thus, the model biofilms showed an increase in resistance compared to planktonic cells of the same strain, but the level of resistance to different antibiotics varied greatly. The maximum BRR increase was found with meropenem, which at the same time proved to be highly effective against planktonic cells.

FLIP7 and antibiotics interaction (TTC cell viability assay)

To study the interaction of FLIP7 and antibiotics, we tested the antimicrobial activity of 1827 combinations varying in the type of antibiotic and concentrations of the components in accordance with the standard checkerboard method. The test results are summarized in Table 1 (columns 5–8). FICI values of the *S. aureus* biofilm demonstrated high synergy (FICI <0.25) of FLIP7 with meropenem, amikacin, kanamycin, ampicillin and vancomycin. All these antibiotics were also characterized by a high level of BRR. At the same time, antibiotics with a low BRR level and antiseptic benzalkonium chloride showed much higher FICI values, and correspondingly, weaker interaction with FLIP7.

In *E. coli*, the highest synergy was found for meropenem and cefotaxime, which showed the maximum BRR level, whereas antibiotics with low BRR demonstrated a lack of synergy. Polymyxin B with its evidential BRR growth and lack of interaction with FLIP7 stood alone among the other antimicrobials tested. Meropenem and FLIP7 synergy was also significant in experiments with *P. aeruginosa* and *A. baumannii* biofilms, although in *K. pneumoniae* they interacted at the level of additivity.

Antibiotic fold reduction in the presence of FLIP7 effective concentration (column 8 of Table 1) is another important characteristic of the antimicrobials interaction. The most significant fold reduction was found in *S. aureus* biofilm treated with meropenem, amikacin or kanamycin (over 142–500 times). The fold reduction of other antibiotics and antiseptic benzalkonium chloride varied, ranging from 5 to over 25 times. In *E. coli*, meropenem showed the most pronounced fold reduction (over 188 times). The reduction of $MBIC_{90}$ of other antibiotics was reliable as well, except for polymyxin B. FLIP7 also reduced the effective concentration of meropenem in *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* biofilms, although not as significantly as in *S. aureus* and *E. coli*.

In parallel with experiments in biofilms, the interaction of FLIP7 and antibiotics was studied in planktonic cultures of the same strains (Table 2). In general, the interaction of FLIP7 and antibiotics in planktonic cells was significantly weaker than in biofilms and mainly additive. Decline of the interaction was particularly noticeable in high-BRR antibiotics. The fold reduction analysis demonstrated a similar trend with some exceptions. Thus, polymyxin B showed no interaction with FLIP7 in the *E. coli* biofilm, while with the planktonic culture the interaction was well defined. A more significant level of fold reduction in *E. coli* planktonic cells compared to biofilms was also noted in tetracycline and chloramphenicol. *A. baumannii* exhibited a similar synergy rate in biofilm and planktonic states and an even higher fold reduction level in planktonic cells.

Dose–effect curves demonstrate another important feature of FLIP7 and antibiotics interaction – the ability to eliminate antibiotic-insensitive persister cells, which is especially pronounced in *S. aureus* biofilm (Figure 1). Meropenem caused a dose-dependent decrease in cell survival in the concentration range from 0.1 to 0.8 μg/mL, and further increase in concentration did not lead to a decrease in survival. In accordance with the generally accepted interpretation,[26] this indicates the presence in the population of antibiotic-insensitive persistent cells. The combination of meropenem with an effective concentration of FLIP7 completely eliminated (or reduced to an undetectable level) metabolically active cells, starting with an antibiotic concentration of 0.1 μg/mL. The same effect was observed in experiments with other high-BRR antibiotics: amikacin, ampicillin and kanamycin. With antibiotics with low or zero BRR (oxacillin, chloramphenicol), persistent bacteria were not detected in the biofilm. Antibiotics with an intermediate (10- to 84-fold) level showed the presence in the population of cells with intermediate resistance to vancomycin, benzalkonium chloride and erythromycin (data not shown). *E. coli* dose–effect curves of high-BRR antibiotics meropenem and cefotaxime also revealed the presence of persister cells eliminated by the antibiotic combination with FLIP7, whereas those of the low-BRR antibiotics ciprofloxacin and chloramphenicol did not reveal persister cells (Figure 2). The persisters were not clearly detected in meropenem-treated *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* biofilms (data not shown).

FLIP7 and antibiotics interaction (crystal violet biofilm eradication assay)

The interaction of FLIP7 and antibiotics was also studied by staining the biofilms with crystal violet dye (biofilm eradication assay). In total, 862 combinations organized in accordance with standard checkerboard scheme were tested. The analysis of the FICI and fold reduction values showed results almost identical to the TTC method (Table 3). In *S. aureus* biofilm, FLIP7 demonstrated high levels of synergy

Table 2 FLIP7 and antibiotics interaction on planktonic cells (TTC assay)

Strain/antibiotic	Antibiotic MIC_{90} (μg/mL)	Antibiotic MIC_{90} in FLIP7 presence (μg/mL)[1]	FICI	Interaction type	Fold reduction[2]
Staphylococcus aureus					
Meropenem	0.02 ± 0.02	<0.002	0.667	Add	>10
Amikacin	1.88 ± 0.00	0.36 ± 0.12	0.833	Add	5.2
Kanamycin	3.51 ± 1.17	<0.1	0.527	Add	>35.1
Ampicillin	0.03 ± 0.01	0.007 ± 0.001	0.542	Add	4.3
Vancomycin	0.41 ± 0.19	0.12 ± 0.00	0.589	Add	3.4
Benzalkonium Cl	0.30 ± 0.00	0.15 ± 0.00	0.750	Add	2.0
Clindamycin	75.0 ± 0.0	4.70 ± 0.00	0.729	Add	15.9
Erythromycin	0.94 ± 0.00	0.03 ± 0.00	0.589	Add	31.3
Oxacillin	0.05 ± 0.00	0.014 ± 0.002	1.042	Ind	3.6
Chloramphenicol	4.70 ± 0.00	0.60 ± 0.00	0.418	Syn	7.8
Escherichia coli					
Meropenem	0.03 ± 0.00	0.03 ± 0.00	1.085	Ind	1.0
Cefotaxime	0.09 ± 0.00	0.02 ± 0.00	0.415	Syn	4.5
Polymyxin B	0.34 ± 0.11	<0.02	0.339	Syn	17.0
Ciprofloxacin	0.03 ± 0.00	0.015 ± 0.00	0.666	Add	2.0
Tetracycline	0.46 ± 0.00	0.03 ± 0.00	0.729	Add	15.3
Gentamicin	4.69 ± 0.00	3.53 ± 1.17	1.085	Ind	1.3
Chloramphenicol	4.69 ± 0.00	0.30 ± 0.00	0.670	Add	15.6
Pseudomonas aeruginosa					
Meropenem	0.94 ± 0.31	0.48 ± 0.00	0.516	Add	2.0
Klebsiella pneumoniae					
Meropenem	0.01 ± 0.00	0.01 ± 0.00	1.042	Ind	1.0
Acinetobacter baumannii					
Meropenem	0.88 ± 0.13	0.03 ± 0.01	0.393	Syn	29.3

Notes: [1]MIC_{90} of antibiotic in combination with FLIP7 concentration causing maximum antibiotic fold reduction. [2]Ratio of antibiotic MIC_{90} in the absence and presence of FLIP7 concentration causing maximum antibiotic fold reduction. Data presented as mean ± SE.

Abbreviations: TTC, trimethyl tetrazolium chloride; MIC, minimum inhibiting concentration; FICI, fractional inhibitory concentration index; Syn, synergy; Add, additivity; Ind, independence.

and fold reduction with meropenem, amikacin and kanamycin and a lower level in combination with chloramphenicol. In *E. coli*, the interaction with meropenem and cefotaxime was synergistic, whereas in combination with ciprofloxacin the effect was only additive. Synergy with meropenem was also found in *P. aeruginosa* and *A. baumannii*, while the interaction was only additive in *K. pneumoniae*.

Analysis of the dose–effect curves showed that meropenem, amikacin and kanamycin are not capable of destroying the *S. aureus* biofilm material, although they almost completely eradicated the material in the presence of an effective FLIP7 concentration (Figure 3). In contrast to these antibiotics, chloramphenicol effectively destroyed the biofilm material at a concentration of 2 μg/mL and more. Under these conditions, combination with FLIP7 enhanced the effect of the antibiotic in the range of its low concentrations. In experiments with *E. coli* (Figure 4), similar results were obtained: FLIP7 drastically improved the limited ability of meropenem and cefotaxime to destroy biofilm material and strengthened the action of subthreshold concentrations of ciprofloxacin. Potentiation of the effect of meropenem was also well expressed in *P. aeruginosa* and *A. baumannii* biofilms and less considerable although reliable in *K. pneumoniae*.

Biofilm microscopic visualization

In addition to obtaining the quantitative data of the TTC and crystal violet tests, changes in the biofilm state were visualized at the level of light microscopy. An *S. aureus* biofilm treated with meropenem alone (0.2 μg/mL) or the same concentration of meropenem and FLIP7 (125 μg/mL) was chosen as the model. High-synergy concentrations of the active substances were selected based on the results of the checkerboard experiments discussed above. The data of the TTC and crystal violet tests showed that this combination causes complete suppression of metabolic activity and destruction of the biofilm material, respectively. Based on this, it was expected that processing the biofilm with the combination would cause the killing of living cells and biofilm material

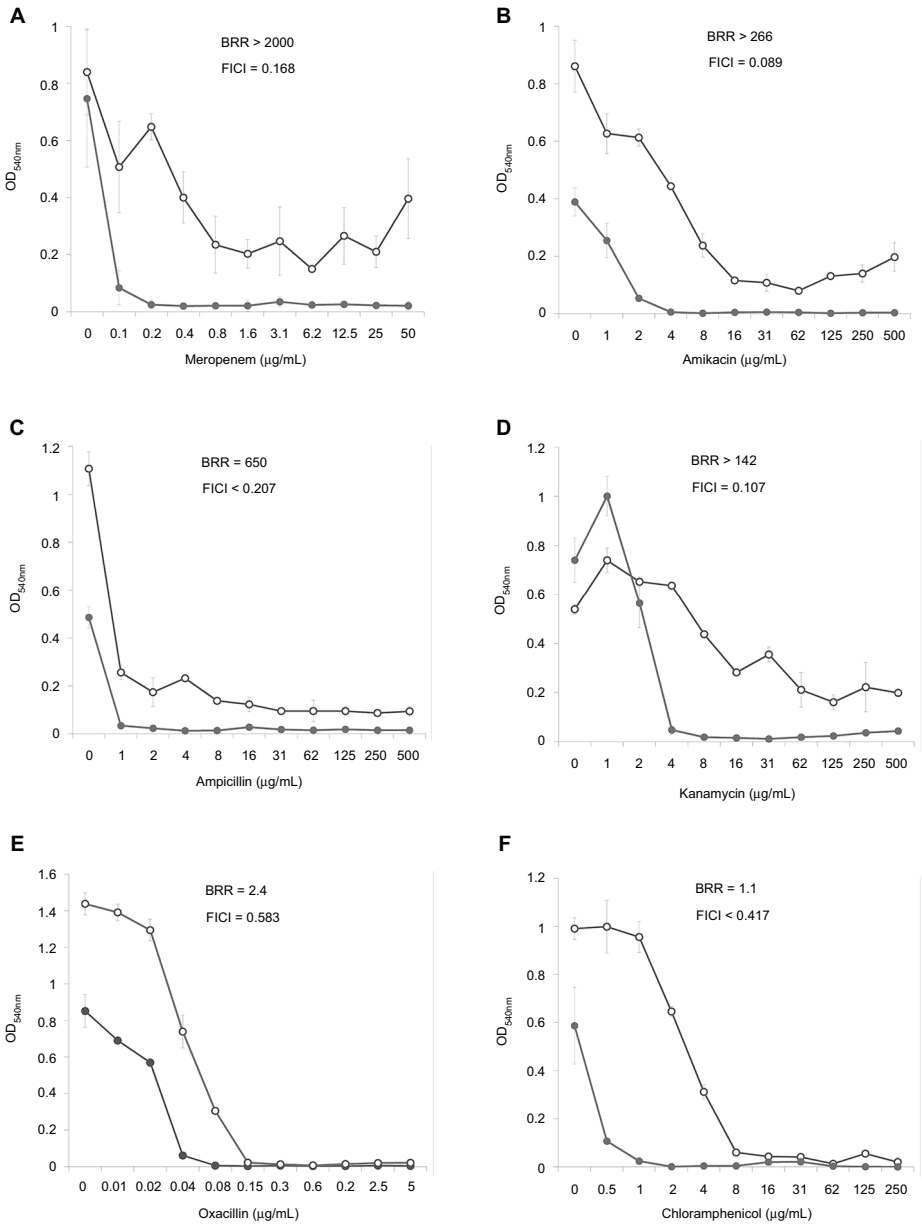

Figure 1 Doze–effect curves of antibiotics alone and in combination with FLIP7 against *Staphylococcus aureus* biofilm (TTC assay).
Notes: (**A**) Meropenem versus meropenem + FLIP7 125 μg/mL. (**B**) Amikacin versus amikacin + FLIP7 250 μg/mL. (**C**) Ampicillin versus ampicillin + FLIP7 125 μg/mL. (**D**) Kanamycin versus kanamycin + FLIP7 250 μg/mL. (**E**) Oxacillin versus oxacillin + FLIP7 250 μg/mL. (**F**) Chloramphenicol versus chloramphenicol + FLIP7 125 μg/mL. Open circles – antibiotic, closed circles – the antibiotic + FLIP7; Y-axis – optical density (mean ± standard error). Effective concentrations of FLIP7 were individually determined for each antibiotic based on the checkerboard experiments.
Abbreviations: TTC, trimethyl tetrazolium chloride; OD, optical density; BRR, biofilm-related resistance; FICI, fractional inhibitory concentration index.

destruction, which could be microscopically observed. The results of the microscopic analysis corresponded well to the expectations (Figure 5). In the control, bacteria formed a multilayered biofilm that was firmly attached to a glass surface and consisted of cells of regular round shape with no signs of damage. Treatment with meropenem alone led to a strong thinning of the population represented mainly by damaged cells of irregular shape, although apparently normal cells (presumable persisters) were also present in the biofilm. After treatment with the combination, only a small number of dead or obviously damaged "ghost" cells remained on the glass. No evidence of live intact cells was observed with this treatment option. Thus, microscopic examination provided independent confirmation of the results of spectrophotometric analyses.

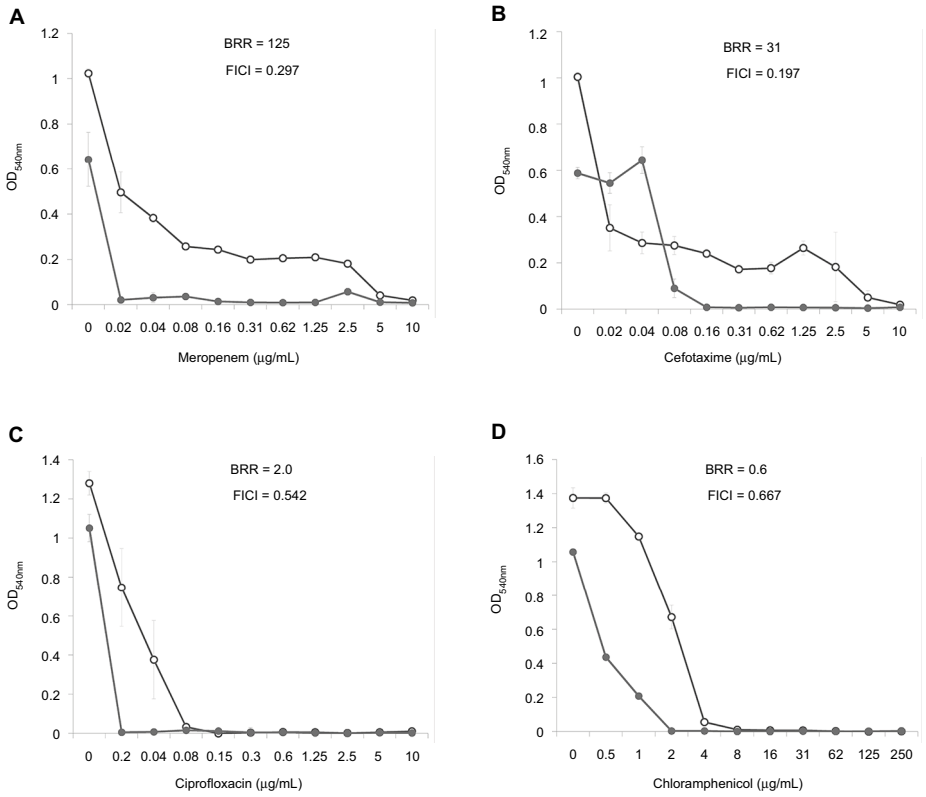

Figure 2 Doze–effect curves of antibiotics alone and in combination with FLIP7 against *Escherichia coli* biofilm (TTC assay).
Notes: (**A**) Meropenem versus meropenem + FLIP7 500 μg/mL. (**B**) Cefotaxime versus cefotaxime + FLIP7 500 μg/mL. (**C**) Ciprofloxacin versus ciprofloxacin + FLIP7 500 μg/mL. (**D**) Chloramphenicol versus chloramphenicol + FLIP7 250 μg/mL.
Abbreviations: TTC, trimethyl tetrazolium chloride; OD, optical density; BRR, biofilm-related resistance; FICI, fractional inhibitory concentration index.

Table 3 FLIP7 and antibiotics interaction in crystal violet biofilm eradication assay

Strain/antibiotic	FICI	Interaction type	Antibiotic $MBEC_{90}$ (μg/mL)	Antibiotic $MBEC_{90}$ in FLIP7 presence (μg/mL)[1]	Fold reduction[2]
Staphylococcus aureus					
Meropenem	0.169	Syn	>50	0.15 ± 0.0	>333
Amikacin	0.024	Syn	>500	1.5 ± 0.0	>333
Kanamycin	0.137	Syn	>500	3.5 ± 0.5	>143
Chloramphenicol	0.438	Syn	3.0 ± 1.0	<0.5	>6
Escherichia coli					
Meropenem	0.349	Syn	2.19 ± 0.31	0.03 ± 0.0	73
Cefotaxime	0.397	Syn	1.56 ± 0.31	0.1 ± 0.02	16
Ciprofloxacin	0.583	Add	0.06 ± 0.0	<0.02	>3
Pseudomonas aeruginosa					
Meropenem	0.282	Syn	9.4 ± 0.0	2.36 ± 0.0	4
Klebsiella pneumoniae					
Meropenem	0.624	Add	6.25 ± 0.0	1.17 ± 0.0	5
Acinetobacter baumannii					
Meropenem	0.291	Syn	9.4 ± 0.0	1.37 ± 0.19	7

Notes: [1]$MBEC_{90}$ of antibiotic in combination with FLIP7 concentration causing maximum antibiotic fold reduction. [2]Ratio of antibiotic $MBEC_{90}$ in the absence and presence of FLIP7 concentration causing maximum antibiotic fold reduction. Data presented as mean ± SE.
Abbreviations: FICI, fractional inhibitory concentration index; MBEC, minimum biofilm eradication concentration; Syn, synergy; Add, additivity.

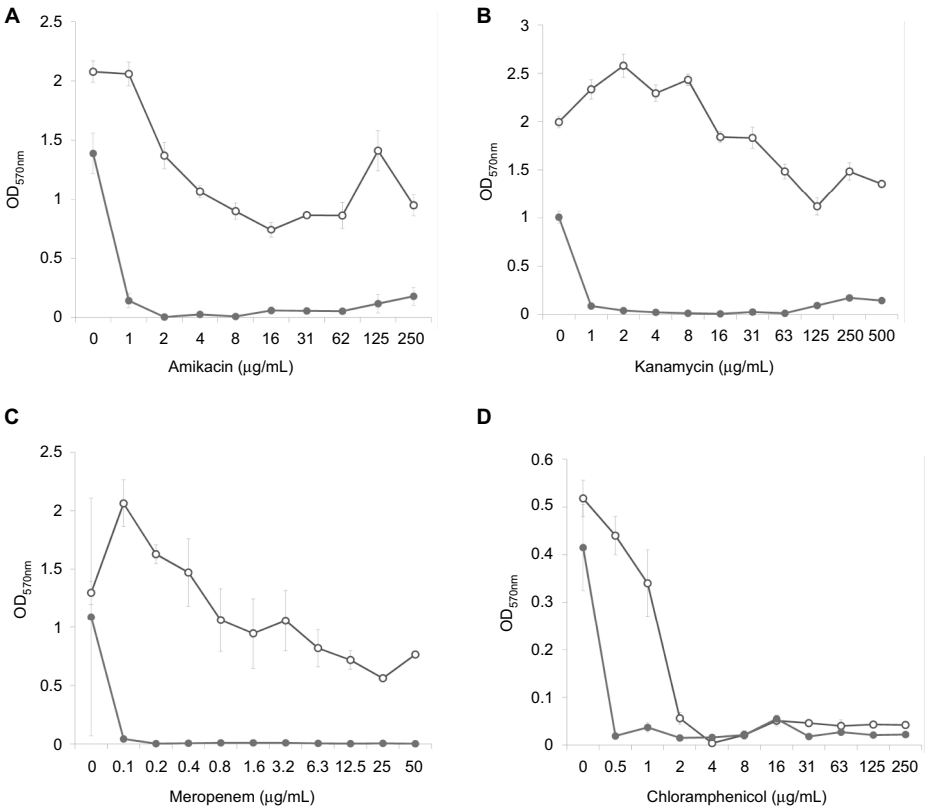

Figure 3 Doze–effect curves of antibiotics alone and in combination with FLIP7 against *Staphylococcus aureus* biofilm (crystal violet assay).
Notes: (**A**) Amikacin versus amikacin + FLIP7 500 μg/mL. (**B**) Kanamycin versus kanamycin + FLIP7 500 μg/mL. (**C**) Meropenem versus meropenem + FLIP7 250 μg/mL. (**D**) Chloramphenicol versus chloramphenicol + FLIP7 250 μg/mL. Open circles: antibiotic; closed circles: antibiotic + FLIP7.
Abbreviation: OD, optical density.

Discussion

Along with the growing prevalence of antibiotic-resistant strains, BRR represents one of the two main obstacles in the fight against bacterial infections. There are different approaches to the treatment of biofilms in the research stage, but the problem remains unresolved so far.[14,15,27]

While studying insect immunity, we noted that insects respond to infection with synchronous synthesis of not one but a handful of AMPs. A particularly rich repertoire and a high concentration of AMPs were found in the hemolymph of bacteria-challenged maggots of the blue blowfly *C. vicina*. The *C. vicina* AMP complex comprises four AMP families: defensins, cecropins, diptericins and proline-rich peptides.[23,24] AMPs similar in structure and functions were found in various combinations in many other insects.[28,29] Defensins are peptides with a three-dimensional structure containing α-helix/β-sheet elements coordinated by three disulfide bridges and predominantly active against Gram-positive bacteria by means of bacterial cell wall disruption/permeabilization. Cecropins are linear amphipathic α-helical peptides particularly active against Gram-negative bacteria and known to have pore-forming and cell membrane-permeabilizing activity. Diptericins are members of a glycine-rich AMP family selectively toxic to some Gram-negative bacteria by means of cell wall disruption. *Calliphora* proline-rich peptides belong to the family of proline-/arginine-rich AMPs. In contrast to defensins, cecropins and diptericins, proline-/arginine-rich AMPs kill bacteria by damaging DNA and/or protein synthesis. Thus, FLIP7 comprises three structurally distinct families of cell wall-disrupting AMPs targeted predominantly to the membranes of Gram-negative (cecropins, diptericins) or Gram-positive (defensins) bacteria and one group affecting intracellular targets (proline-rich peptides). From a mechanistic point of view, the composition looks like a well-organized system of complementary elements the combined action of which protects the host organism from various potentially dangerous bacteria.

In the current work, we studied the interaction of FLIP7 and antibiotics using several in vitro models of mature biofilms and planktonic cultures. As expected, bacteria in the biofilm

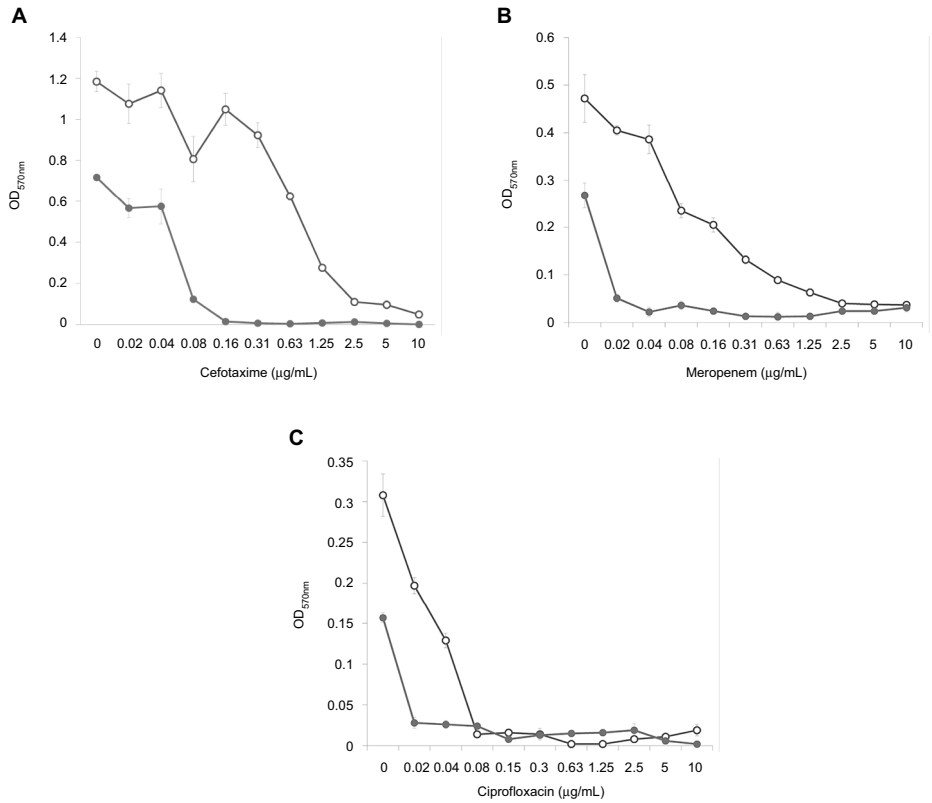

Figure 4 Doze–effect curves of antibiotics alone and in combination with FLIP7 against *Escherichia coli* biofilm (crystal violet assay).
Notes: (**A**) Cefotaxime versus cefotaxime + FLIP7 500 μg/mL. (**B**) Meropenem versus meropenem + FLIP7 500 μg/mL. (**C**) Ciprofloxacin versus ciprofloxacin + FLIP7 250 μg/mL. Open circles: antibiotic; closed circles: antibiotic + FLIP7.
Abbreviation: OD, optical density.

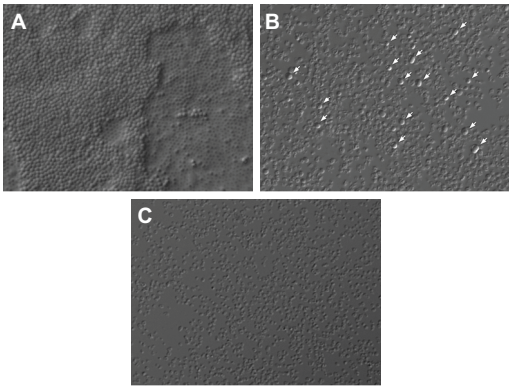

Figure 5 *Staphylococcus aureus* biofilm microscopic visualization (Nomarski optics ×1000).
Notes: Twenty-four-hour biofilms were formed on the coverslip and processed as follows: (**A**) control; (**B**) treated with meropenem 0.2 μg/mL; and (**C**) treated with meropenem 0.2 μg/mL + FLIP7 125 μg/mL. In control, bacteria formed a multilayered biofilm consisting of live cells of regular shape. Treatment with meropenem killed most of the bacteria but a portion of the cells (presumable persisters) survived (survivors are marked with arrows). After treatment with the combination, no signs of living cells were seen on the glass.

state showed increased recalcitrance to antibiotics compared to planktonic cells of the same strain. However, the level of BRR differed greatly, depending on the type of antibiotic and the species of microorganism. The *S. aureus* biofilm became almost completely insensitive to the beta-lactams meropenem and ampicillin, and aminoglycosides amikacin and kanamycin, which effectively killed planktonic cells. An intermediate BRR level was found for vancomycin, erythromycin and antiseptic benzalkonium chloride, while for other antibiotics (oxacillin, tetracycline and chloramphenicol) BRR was weakly expressed. Varying BRR was found for the *E coli* biofilm as well.

Checkerboard experiments with biofilm models demonstrated the positive interaction of FLIP7 with almost all of the antibiotics in all bacteria studied except polymyxin B in *E. coli*. In *S. aureus*, high synergy (FICI <0.25) was observed with five antibiotics of the 11 tested (Table 1). In publications concerning *S. aureus* biofilms, we were able to find the FICI values of 37 diverse two-component AMP–antibiotic combinations.[30–32] FICIs <0.25 were registered only in two cases: cecropin A synthetic analog + ciprofloxacin[30] and peptide 1018 + ceftazidime.[32] Comparison with the literature data showed that a high synergy with antibiotics is much more common in FLIP7 than in individual AMPs referenced above (45% versus 5%, $P = 0.005$, Z-test)

The interaction in planktonic cell models was, in most cases, significantly weaker than in biofilms of the same strains and never reached a high synergy level. This observation is in agreement with the fact that a high synergy level was only found in high-BRR antibiotics. If the antibiotic did not encounter a BRR barrier (planktonic cells) or the barrier was weak (low-BRR antibiotics), the interaction strength was greatly reduced. From this point of view, FLIP7 can be considered a synergist helping antibiotics to overcome the BRR barrier.

The analysis of the dose–effect curves points to persister cells as a likely target of FLIP7 synergistic effect. It is known that the biphasic character of the curve indicates the presence in the population of persisters insensitive to the antibiotic.[33] In the *S. aureus* biofilm, dose–effect curves of high-BRR/high-synergy antibiotics (meropenem, ampicillin, amikacin and kanamycin) had a typical biphasic shape (Figure 1). The combination of these antibiotics with FLIP7 resulted in complete elimination of survivors detectable by TTC method, while the antibiotic alone could not cope with this task. The dose–effect curves of antibiotics with a minimal BRR level (oxacillin, chloramphenicol) did not reveal persister cells in the biofilm population. A similar pattern was observed in the *E. coli* biofilm: persisters were suppressed by the combination of FLIP7 and antibiotic in all cases when such cells were found in the population (Figure 2).

However, the TTC test can only detect metabolically active cells and may overlook the persisters. Microscopic analysis of the *S. aureus* biofilm treated with a highly synergistic combination of FLIP7 and meropenem confirmed TTC test data (Figure 5). After the treatment, we were unable to detect living cells, although they were visible in the biofilm treated with meropenem alone. Demonstration of this fact confirms the possibility of complete elimination of bacterial cells in the biofilm by a combination of FLIP7 and antibiotic and thus preventing the recurrence of the disease.

The biofilm eradication assay revealed another possible mechanism of the interaction. In this test, the crystal violet binding is directly proportional to the total content of all biofilm components (extracellular matrix, dead and living cells). The binding of the dye was sharply reduced after the treatment of *S. aureus* and *E. coli* biofilms with the combination of FLIP7 and antibiotics in comparison with the antibiotic alone (Figures 3 and 4). Similar results were obtained in the study of meropenem action on *P. aeruginosa* and *A. baumannii* biofilms, even though the effect was less pronounced in *K. pneumoniae*. The total destruction of the biofilm evidently means the elimination of the extracellular matrix, which, along with persisters, plays a key role in protecting biofilms from antibiotic assault. Thus, destruction of the matrix is another possible mechanism for the interaction of FLIP7 and antibiotics.

In summary, it can be concluded that FLIP7 is a highly efficient natural antimicrobial system helping antibiotics to overcome strong biofilm barriers through persisters' sensitization and biofilm material destruction. The system comprises four AMP families with distinct structures and mechanisms of action. Inevitably, the question arises whether it is technically and economically feasible to use such a complex system in medical practice. Even if it is theoretically possible to synthesize all the active components of the complex, the manufacturing cost of such a man-made composition is likely to be unacceptably high. Therefore, we opted for biotechnology for the parallel biosynthesis of natural AMPs in a *C. vicina* culture. The technology allows scalable cost-effective production of the AMPs, the composition of which is identical to natural FLIP7.[34]

Another problem comes with choosing an appropriate FLIP7 partner among conventional antibiotics. Judging from the results of the study, the standard checkerboard method is well suited for this purpose. At present, beta-lactams meropenem and ampicillin, aminoglycosides amikacin and kanamycin, glycopeptide vancomycin and cephalosporin cefotaxime look like the most suitable candidates in respect to the synergy level.

Currently, these and many other antibiotics are being used successfully to treat early stages of infections. At later stages accompanied by the emergence of biofilms, their use is much less effective even with the greatest possible increase in dosage. The synergistic effect of FLIP7 allows reducing the effective anti-biofilm concentration of antibiotic to a level well below the one clinically achievable (Table 4). From this point of view, the best partners of FLIP7 in the treatment of *S. aureus* and/or *E. coli* biofilms are meropenem, ampicillin, oxacillin, ciprofloxacin and cefotaxime the effective anti-biofilm concentrations of which can be reduced by 2–3 orders of magnitude compared with clinically achievable concentrations. The acceptable width of the pharmacological window is also characteristic of vancomycin, amikacin, gentamicin and tetracycline where the clinically achievable concentration exceeds that required for destruction of the biofilm by >20 times. Biofilms of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* can also be sensitized to the action of the antibiotic with the help of FLIP7; however, the data available now are limited to the example of meropenem.

In evaluating the clinical prospects of FLIP7, attention should be paid to the totality of its properties that are well

Table 4 Pharmacological windows of antibiotics used in combination with FLIP7

Antibiotic	Clinically achievable concentration[1]		Pharmacological window[2]	
	µg/mL	Reference		
			Staphylococcus aureus	>300
			Escherichia coli	>1500
Meropenem	40	35	*Klebsiella pneumoniae*	125
			Acinetobacter baumannii	25
			Pseudomonas aeruginosa	12.5
Ampicillin	281	36	*S. aureus*	>281
Cefotaxime	41	37	*E. coli*	586
Oxacillin	64	38	*S. aureus*	6400
Vancomycin	53	39	*S. aureus*	35
Amikacin	45	40	*S. aureus*	30
Kanamycin	16	41	*S. aureus*	5.5
Gentamicin	14	42	*E. coli*	23
Erythromycin	6.5	43[3]	*S. aureus*	14
Ciprofloxacin	6.7	44	*E. coli*	>335
Tetracycline	5	45	*S. aureus*	>50
			E. coli	33
Chloramphenicol	20	46	*S. aureus*	20
			E. coli	13

Notes: [1]Plasma C_{max} at intravenous administration. [2]The ratio of clinically achievable concentration and $MBIC_{90}$ of the antibiotic in the presence of FLIP7. The values of $MBIC_{90}$ are taken from Table 1. [3]Plasma C_{max} at oral administration.
Abbreviation: MBIC, minimum biofilm-inhibiting concentration.

suited to the tasks of treating biofilm infections: efficacy against planktonic and biofilm bacteria of various species; sensitization of persisters to the action of antibiotics; the ability to destroy biofilm material; high synergy with antibiotics of different classes. The ability to prevent acquired resistance development in Gram-negative bacteria[23] and low toxicity for human cells[24] supplement the list of advantages of FLIP7 as a platform for the future development of new antibacterial drugs.

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Disclosure

SC and NG are inventors of patent RU2447896 "Antimicrobial material" (patentee Allopharm company). SC is a shareholder of Allopharm company. DT and AY have no conflicts of interests to declare in this work.

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