Medical Microbiology and Immunology (2019) 208:877–883
https://doi.org/10.1007/s00430-019-00624-7

RAPID COMMUNICATION

In vitro activity of Protegrin-1, alone and in combination with clinically useful antibiotics, against *Acinetobacter baumannii* strains isolated from surgical wounds

Gianluca Morroni[1] · Oriana Simonetti[2] · Andrea Brenciani[3] · Lucia Brescini[1] · Wojciech Kamysz[4] · Elzbieta Kamysz[5] · Damian Neubauer[4] · Miriam Caffarini[6] · Monia Orciani[6] · Eleonora Giovanetti[7] · Annamaria Offidani[2] · Andrea Giacometti[1] · Oscar Cirioni[1]

Received: 22 October 2018 / Accepted: 7 June 2019 / Published online: 18 June 2019
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Abstract

In the past few years the increasing incidence of hospital infections with *Acinetobacter baumannii*, especially in immunocompromised patients, and its proneness to develop multidrug resistance have been raising considerable concern. This study examines the antimicrobial and antibiofilm activity of protegrin 1 (PG-1), an antimicrobial peptide from porcine leukocytes, against *A. baumannii* strains isolated from surgical wounds. PG-1 was tested both alone and combined with the antibiotics commonly used in clinical settings. Its antimicrobial activity was evaluated by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), checkerboard assays, and time-kill experiments. Its effects on biofilm inhibition/eradication were tested with crystal violet staining. The strains were grown in subinhibitory or increasing PG-1 concentrations to test the development of resistance. Mammalian cell toxicity was tested by XTT assays. PG-1 MICs and MBCs ranged from 2 to 8 µg/ml. PG-1 was most active and demonstrated a synergistic interaction with colistin, a last resort antibiotic. Interestingly, antagonism was never observed. In time-kill experiments, incubation with 2 × MIC for 30 min suppressed all viable cells. PG-1 did not select resistant strains and showed a limited effect on cell viability, but it did exert a strong activity against multidrug-resistant *A. baumannii*. In contrast, in our experimental conditions it had no effect on biofilm inhibition/eradication. PG-1 thus seems to be a promising antimicrobial agent against multidrug-resistant Gram-negative infections.

Keywords *Acinetobacter baumannii* · Antimicrobial peptides · Protegrin-1 · Synergy · Checkerboard assay

Introduction

Multidrug-resistant (MDR) bacteria are a major and growing problem, accounting for an annual toll of about 700,000 deaths and for healthcare costs in excess of $20 billion in the United States alone [1]. Gram-negative bacteria raise

Edited by: Volkhard A. J. Kempf.

Gianluca Morroni and Oriana Simonetti have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00430-019-00624-7) contains supplementary material, which is available to authorized users.

✉ Andrea Brenciani
a.brenciani@univpm.it

[1] Infectious Diseases Clinic, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

[2] Dermatological Clinic, Polytechnic University of Marche, Ancona, Italy

[3] Microbiology Unit, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

[4] Faculty of Pharmacy, Medical University of Gdansk, Gdańsk, Poland

[5] Faculty of Chemistry, University of Gdansk, Gdańsk, Poland

[6] Histology, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Ancona, Italy

[7] Microbiology Unit, Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy

the most severe concern, due to their increasing resistance to traditional antibiotics, the spread of extended-spectrum β-lactamases and carbapenemases, and the increasing diffusion of *mcr* genes, which are responsible for resistance to colistin, a last resort antibiotic [2]; in particular, *Acinetobacter baumannii*, formerly considered a low-grade pathogen, is a cause for great concern—especially where hospital-acquired infections and immunodeficient patients are involved—due to its proneness to acquire a wide range of antibiotic resistance mechanisms [3]. The incidence of nosocomial infections with *A. baumannii* has been growing all over the world, especially in intensive care units, and has reached about 1 million cases a year [4]. Given the increasing bacterial resistance also to last resort antibiotics like colistin and tigecycline [5, 6], new antimicrobials are clearly needed.

In the past few years, research into novel and unconventional approaches to treating MDR bacterial infections has focused on antimicrobial peptides (AMPs), small molecules (< 10 kDa) produced by all living species. AMPs are divided into four structural classes (α-helical, β-sheet, loop, and extended peptides) and show a wide range of biological activities including antimicrobial [7], immunomodulatory and antibiofilm actions [8].

Protegrins are β-sheet peptides belonging to the cathelicidin family, which exert broad-spectrum antimicrobial activity. Of the five known protegrins, the most abundant and best characterised is protegrin 1 (PG-1), an 18-amino acid peptide with a β-hairpin structure stabilised with two disulphide bridges [9]. Although the antibacterial activity of PG-1 has been tested against a variety of bacteria [10, 11], there are no data on its effectiveness against *A. baumannii*.

This work was devised to test the antibacterial and antibiofilm activity of PG-1 against MDR strains of *A. baumannii* isolated from surgical wounds and its possible synergistic effect with the antibiotics commonly used in clinical settings.

Materials and methods

Antimicrobial peptide and antibiotics

PG-1 (RGGRLCYCRRRFCVCVGR-NH_2 with two disulphide bridges, Cys6-Cys15 and Cys8-Cys13) was synthesized as described previously [9]. Colistin, fosfomycin, levofloxacin, meropenem, tigecycline, and rifampin (all purchased from Sigma-Aldrich, St. Louis, MO, USA) were used in combination experiments with PG-1.

A. baumannii strains and typing

The bacterial strains included 15 colistin-susceptible and 4 colistin-resistant strains isolated from surgical wounds as well as *A. baumannii* ATCC 19606. Identification and susceptibility tests were performed respectively by MALDI-TOF analysis and the VITEK II system (bioMérieux, Marcy-l'Etoile, France) (Table 1). Strains were typed by ApaI-PFGE and MLST using the Oxford scheme (www.pubmlst.net).

Determination of the minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of colistin, fosfomycin, levofloxacin, meropenem, amikacin, minocycline, doxycycline, tigecycline, and rifampin were confirmed using the broth microdilution method and interpreted according to CLSI guidelines. PG-1 susceptibility was tested by broth microdilution in Mueller–Hinton Cation Adjusted Broth (CAMHB, Liofilchem, Roseto degli Abruzzi, Italy). The minimum bactericidal concentrations (MBCs) were evaluated as described previously [12].

Induction of colistin resistance

A colistin-resistant *A. baumannii* ATCC 19606 mutant was obtained as described by Nhu et al. [13]. MIC determination and ApaI-PFGE typing were performed as described above.

Synergy studies

Interactions were tested in triplicate by a checkerboard assay using 96-well polypropylene microtiter plates. The fractional inhibitory concentration (FIC) index was interpreted as follows: ≤ 0.5, synergy; 1 ≤ to < 4.0, indifference; ≥ 4.0, antagonism [12]. Trimethoprim/Ampicillin combination was used as antagonistic control.

Time-kill assays

Six strains (#238719, #361823, #378648, #379385, #379622 and #12834) representing the six sequence types (STs) determined in the 19 isolates by MLST were used in time-kill assays to evaluate the effect of PG-1 (1×, 2×, 4×, and 8× MIC) and to confirm the synergies demonstrated by the checkerboard assay (1× MIC). The experiments were performed in triplicate using CAMHB, starting from an inoculum of 1×10^8 CFU/ml.

Table 1 Characteristics of the *A. baumannii* strains used

Strain	MIC												MBC	Typing	
	CTX	IMP	MEM	CN	AMK	CIP	COL	SXT	MIN	DOX	TGC	PG-1	PG-1	PFGE-pulsotypes	MLST
238719	> 64	> 16	> 16	> 16	> 64	> 4	0.25	> 16/304	16	32	2	4	4	A	ST218
361823	> 64	> 16	> 16	< 1	2	> 4	0.5	> 16/304	4	32	1	4	4	B1	ST451
371484	> 64	> 16	> 16	> 16	64	> 4	0.25	> 16/304	8	32	0.5	2	2	B2	ST451
371981	> 64	> 16	> 16	4	> 64	> 4	0.25	> 16/304	4	16	2	4	4	B3	ST451
378177	> 64	> 16	> 16	< 1	2	> 4	0.25	> 16/304	4	32	1	4	4	C1	ST281
378648	> 64	> 16	> 16	> 16	> 64	> 4	0.5	> 16/304	1	8	1	8	8	C1	ST281
379385	> 64	> 16	> 16	> 16	> 64	> 4	0.5	> 16/304	4	32	2	8	8	D	ST369
379622	> 64	> 16	> 16	> 16	> 64	> 4	0.125	> 16/304	16	32	2	4	4	E	ST425
380023	> 64	> 16	> 16	> 16	> 64	> 4	0.125	> 16/304	2	8	1	8	8	F	ST369
380667	> 64	> 16	> 16	2	4	> 4	0.5	> 16/304	8	32	4	4	8	E	ST425
381577	> 64	> 16	> 16	< 1	2	> 4	0.06	> 16/304	8	32	2	4	8	G	ST451
382933	> 64	> 16	> 16	> 16	> 64	> 4	0.06	8/152	0.5	1	2	4	4	H	ST281
383074	> 64	> 16	> 16	> 16	> 64	> 4	0.25	8/152	0.5	1	2	4	4	I	ST369
383290	> 64	> 16	> 16	> 16	> 64	> 4	0.125	> 16/304	8	32	< 0.5	8	8	H	ST281
386052	> 64	> 16	> 16	> 16	> 64	> 4	0.25	> 16/304	16	32	2	4	4	L	ST369
388538	> 64	> 16	> 16	> 16	> 64	> 4	128	> 16/304	1	8	1	8	8	C2	ST281
5615	> 64	> 16	32	> 4	> 64	> 4	64	> 16/304	2	1	1	8	8	M	ST281
12316	> 64	> 16	> 64	> 4	> 64	> 4	16	> 16/304	16	16	0.5	8	8	N	ST369
12834	> 64	> 16	32	> 4	> 64	> 4	4	> 16/304	16	32	1	4	4	O	ST348

MICs and MBCs expressed as µg/ml

CTX cefotaxime, *IMP* imipenem, *MEM* meropenem, *CN* gentamicin, *AMK* amikacin, *CIP* ciprofloxacin, *COL* colistin, *SXT* trimethoprim-sulfamethoxazole, *MIN* minocycline, *DOX* doxycycline, *TGC* tigecycline, *FOS* fosfomycin

Biofilm assays

Biofilm quantification experiments were performed in triplicate by crystal violet staining [12] using the six representative strains and *A. baumannii* ATCC 19606. Biofilms were established in 96-well plates containing Brain Heart Infusion Broth (BHIB, Liofilchem) and 1% glucose. PG-1 (0.5× MIC) was added to the broth to test its effect on biofilm formation, whereas its activity on established biofilms was evaluated by adding supra-MIC concentrations (25, 50, and 100 µg/ml) diluted in BHIB/1% glucose for 24 h.

Induction of PG-1 resistance

The development of PG-1 resistance was tested by two approaches. In one experiment, *A. baumannii* ATCC 19606 was grown in 5 ml CAMHB supplemented with 0.5 µg/ml PG-1; 50 µl of overnight culture was daily inoculated in fresh CAMHB containing 0.5 µg/ml PG-1. After 7 and 14 passages, strains were isolated on MacConkey agar (Liofilchem) and the MIC/MBC determined as described previously [12]. In the other experiment, *A. baumannii* ATCC 19606 was incubated in doubling PG-1 concentrations, starting from 0.5 µg/ml, until no growth was detected.

Cytotoxicity experiments

For the cytotoxicity assay, 20,000 HeLa ATCC CCL-2 cells were seeded in 96-well plates and incubated with 10, 25, 50, 100, 200 µg/ml PG-1 for 24 h or 48 h. Cytotoxicity was evaluated with the TACS XTT cell proliferation/viability assay (Trevigen, Italy). HeLa ATCC CCL-2 cells cultured without PG-1 for 24 h or 48 h were used as controls. Data are reported as percentage (mean ± standard deviation) of the values measured in control cells in three independent experiments.

Statistical analysis was performed with Student's *t* test. A p value < 0.05 was considered as statistically significant.

Results

All 19 clinical strains showed multidrug resistance, including resistance to carbapenems. ApaI-PFGE demonstrated 13 different pulsotypes and 6 closely related PFGE patterns, whereas MLST revealed 6 different STs (1 ST218; 6 ST281; 1 ST348; 1 ST369; 2 ST425; and 4 ST451), all belonging to clonal complex 92, the international clone II associated with nosocomial infection. These data are reported in Table 1.

PG-1 was active against all *A. baumannii* strains, both colistin-susceptible and colistin-resistant, at concentrations ranging from 2 to 8 µg/ml. In nearly all strains the MBC was identical to the MIC or it was one dilution higher (Table 1). The MIC_{50} and MIC_{90} were 4 and 8 µg/ml, respectively. PG-1 was also active against *A. baumannii* ATCC 19606 and the isogenic colistin-resistant mutant (colistin MIC, 16 µg/ml); notably, the two strains shared the same MIC/MBC (8 µg/ml).

The drug combination experiments demonstrated a synergistic action of PG-1 and colistin with a FIC index ≤ 0.5 for all strains, including the colistin-resistant isolates (Figure S1). The combinations with fosfomycin, meropenem, and tigecycline also showed a synergistic effect, but not against all strains (respectively 9, 9, and 5 isolates), reflecting a strain-related behaviour. In the checkerboard assays, the combination with levofloxacin or rifampin showed an indifferent effect with all strains. Interestingly, antagonism was never detected. These data are summarised in Table 2.

The synergistic effects were all confirmed by the time-kill assays. PG-1 and colistin were more active combined than alone and induced a faster and complete suppression of cell viability (Fig. 1). In time-kill assays a PG-1 concentration of 8 × MIC inhibited growth in all strains. The lower concentrations mostly induced partial inhibition followed by regrowth (Fig. 2); a concentration of 1 × MIC reduced viable cells by at least 3 Log_{10} after 60 min (complete inhibition was seen only in strain #378648), whereas 2 × and 4 × MIC killed all cells in 1–8 h, depending on the isolate.

PG-1 showed no antibiofilm activity. All *A. baumannii* strains formed biofilm, but PG-1 exposure induced no difference compared with controls. Supra-MIC concentrations also failed to induce a significant reduction of preformed biofilms (Figure S2).

Serial passages in broth containing 0.5 µg/ml PG-1 failed to select resistant strains, since after exposure for 7 and 14 days the MICs and MBCs were the same as in the original strain. Similar results were obtained with *A. baumannii* ATCC 19606 exposed to increasing PG-1 concentrations. No growth was obtained in presence of 8 µg/ml PG-1.

The cytotoxicity assays were negative. Exposure to ≤ 50 µg/ml PG-1 for 24 h or 48 h did not affect cell number, which was comparable to the control culture. Viability was affected at concentrations > 50 µg/ml. The MIC_{50} was > 50 µg/ml (Figure S3).

Discussion

A. baumannii is one of the most common opportunistic pathogens involved in hospital-acquired infections (ESKAPE organisms). Its excellent adaptability and capacity to acquire multiple antibiotic resistance mechanisms are posing an increasing threat.

AMPs are extensively being investigated as antimicrobial agents and some of them, such as pexiganan and iseganan, are undergoing clinical trials [7]. Despite its broad-spectrum activity, data on the efficacy of PG-1, especially against *A. baumannii*, are very limited [10, 11, 14]. The present study documents its good activity against MDR clinical isolates of *A. baumannii* with MICs comparable to that of *A. baumannii* ATCC 19606, and suggests that PG-1 might also be active against other MDR *A. baumannii* strains. Notably, the MIC_{50} and MIC_{90} were comparable with the MICs against *A. baumannii* described for other AMPs [15].

Similar to colistin, the mechanism of action of AMPs involves a negative membrane charge [7]; nonetheless, in this study the mutation conferring colistin resistance did not affect the MIC of PG-1. It is conceivable that the two molecules have different targets and mechanisms of action: whereas colistin binds lipid A of LPS, thus destabilising the outer membrane [16], PG-1 binds the membrane phospholipids, promoting pore formation [17].

Given the shortage of new antibiotics, effective combinations of AMPs and existing antibiotics would provide major advantages; indeed, synergistic actions have already

Table 2 FIC index of each PG-1/antibiotic combination against 19 clinical *A. baumannii* strains, *A. baumannii* ATCC 19606, and its colistin-resistant isogenic mutant strain

Combinations	FIC index (%, range values)		
	Synergy	Indifference	Antagonism
PG-1/Colistin	21/21 (100, 0.182–0.500)	0/21	0/21
PG-1/Fosfomycin	9/21 (42, 0.094–0.500)	12/21 (58, 0.501–2)	0/21
PG-1/Levofloxacin	0/21	21/21 (100, 0.501–1.500)	0/21
PG-1/Meropenem	9/21 (42, 0.251–0.500)	12/21 (58, 0.502–1)	0/21
PG-1/Tigecycline	5/21 (24, 0.252–0.500)	16/21 (76, 0.501–1)	0/21
PG-1/Rifampin	0/21	21/21 (100, 0.501–1)	0/21
Trimethoprim/Ampicillin (antagonistic control)	0/21	0/21	21/21 (100, 4–10)

Results are expressed as number of positive strains/number of strains tested, percentage, and FIC index range

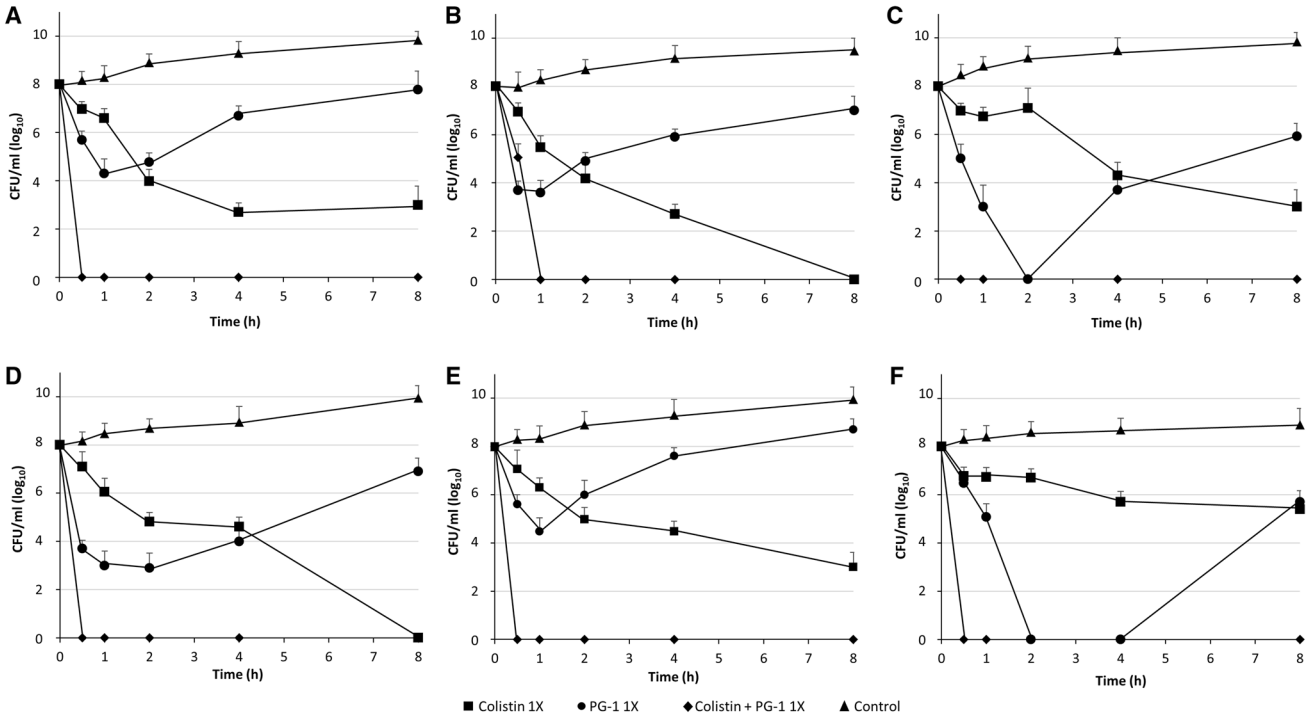

Fig. 1 Time-kill curves of PG-1/colistin against six representative *A. baumannii* strains. **a** #238719 (ST218), **b** #361823 (ST451), **c** #378648 (ST281), **d** #379385 (ST369), **e** #380677 (ST425), **f** #12834 (ST348). Points represent mean values from three independent experiments with standard deviation (SD)

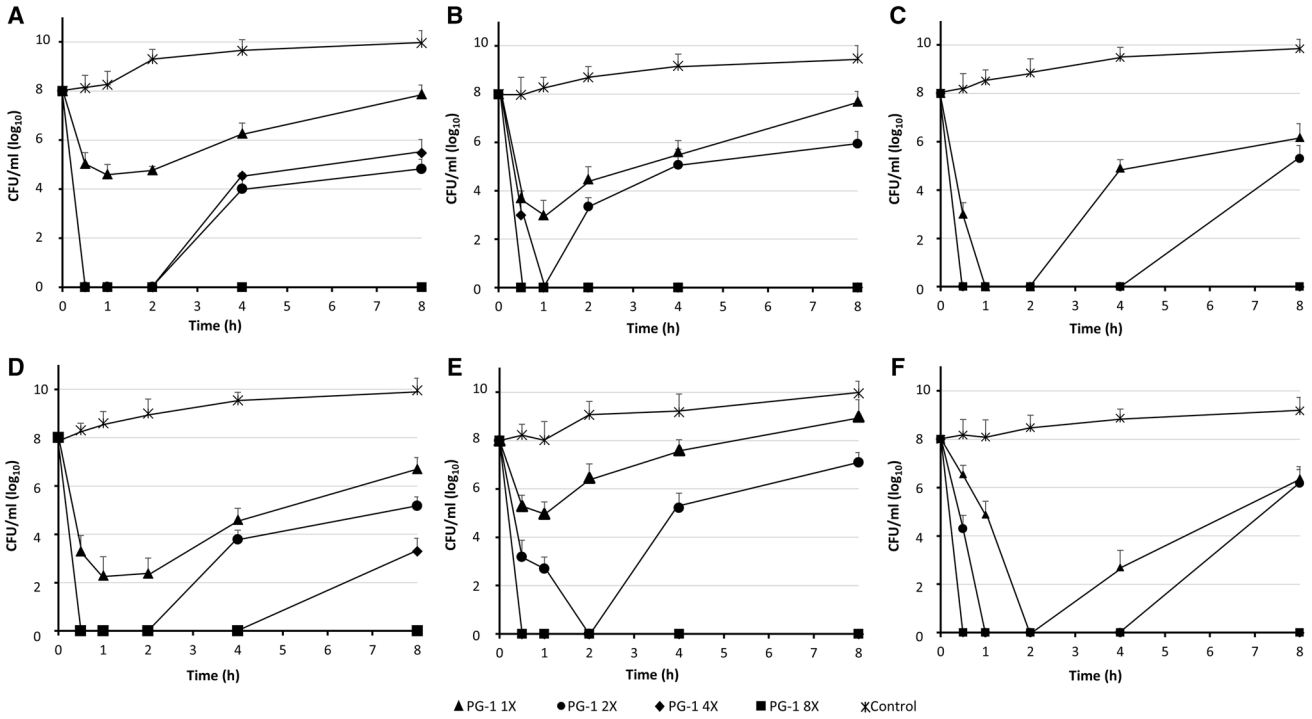

Fig. 2 Time-kill curves of PG-1 against six representative *A. baumannii* strains. **a** #238719 (ST218), **b** #361823 (ST451), **c** #378648 (ST281), **d** #379385 (ST369), **e** #380677 (ST425), **f** #12834 (ST348). Points represent mean values from three independent experiments with SD

been reported, especially with colistin [18, 19]. The present study documented a synergistic effect of PG-1 and colistin also against the colistin-resistant strains. Moreover, since PG-1 demonstrated no antagonism with the antimicrobial agents tested in this work, it is reasonable to hypothesise that it can be used with other antibiotics besides colistin.

The time-kill assays demonstrated that PG-1 was highly active at a high concentration (8 × MIC); lower concentrations exerted temporary effects, since bacterial regrowth was detected after 1–8 h. Similar behaviours have been reported for other AMPs; for instance, a mastoparan concentration of 8 × MIC had bactericidal activity against *A. baumannii*, whereas lower concentrations exerted a transient effect [15].

Some AMPs have been found to have antibiofilm properties and have been denominated antibiofilm peptides [8]. However, in our experimental conditions PG-1 was unable to prevent *A. baumannii* biofilm formation or to disrupt preformed biofilm, even at very high concentrations.

Even though AMP resistance mechanisms have already been described [20], no spontaneous resistant mutants emerged in this study when the *A. baumannii* strains were exposed to subinhibitory or doubling PG-1 concentrations.

The cytotoxicity assays showed that PG-1 had a limited effect on mammalian cells and caused a marked reduction only at concentrations ≥ 50 µg/ml, in line with a previous study where toxicity was demonstrated at ~ 90 µg/ml [14].

The resistance of *A. baumannii* to the common antibiotics is a cause for global concern and stresses the urgent need for new approaches to treat these infections. The present findings indicate that PG-1 is active against MDR clinical *A. baumannii*: it had good in vitro activity and exerted a synergistic effect with colistin while showing no antagonism with the other antibiotics tested; critically, it did not induce resistance in *A. baumannii* in our experimental conditions. Our study included strains solely belonging to CC2 and other experiments should be performed to clarify if PG-1 had activity also against isolates of other clonal complexes. Although further work is clearly needed to establish whether PG-1 can become an antimicrobial agent, these findings indicate that it is a promising molecule.

Acknowledgements We are grateful to Prof. Gian Maria Rossolini and Dr. Alberto Antonelli for providing the colistin-resistant *A. baumannii* strains.

Funding This work was supported by "Progetto Strategico di Ateneo 2016 – UNIVPM: Study of new compounds and innovative strategies to control complicated bacterial skin infections".

Compliance with ethical standards

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

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