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Biocidal Cationic Macromolecules Irrespective of Bacterial Resistance: Our Best Achievements †

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Abstract: Since new antibacterial agents against multi-drug resistant (MDR) bacteria are urgently needed, we recently synthetized cationic dendrimers and copolymers and assessed their antibacterial activity on numerous MDR clinical isolates. Being cationic, the prepared macromolecules electrostatically interacted to pathogens surface, causing irreversible damages and rapid bacterial death. A lysine dendrimer having 192 cationic groups (N^+) was strongly active preferentially on non-fermenting Gram-negative species, displaying MICs comparable to colistin against *P. aeruginosa* (2.1 μM). A lysine dendrimer (128 N^+) was explicitly active on *Acinetobacter*, while a cationic copolymer showed remarkable antibacterial activity against numerous Gram-positive and Gram-negative species. In 24 hour-time-killing, all mentioned macromolecules displayed rapid bactericidal activities. Due to their physicochemical properties, and bactericidal potency, the herein reviewed cationic macromolecules could represent novel tools for realizing either a targeted or a broad-spectrum bactericidal action, regardless the bacterial resistance to current antibiotics, except for colistin.

Keywords: multi drug resistance; new therapeutic options; bactericidal cationic dendrimers; bactericidal cationic copolymers; Gram-positive clinical isolates; Gram-negative strains

1. Introduction

Nowadays, the rapid and worldwide development of multi-drug resistant (MDR) bacteria, responsible of therapeutic failures and growing deaths, is a global concern, urgently requiring efforts to find new curative options [1]. This is also true for opportunistic and nosocomial pathogens, capable to cause severe clinical disorders in immunocompromised and critically ill individuals [1]. The most concern regards Gram-negative bacilli, such as the *Enterobacteriaceae* and the non-fermenting *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Stenotrophomonas maltophylia* species. Such pathogens are emerging as clinically relevant superbugs and are contributing significantly with their worrying levels of resistance to the ineffectiveness of most available antibiotics [2]. However, the antibiotic resistance has also become a major problem in the treatment of infections caused by many Gram-positive bacteria, such as penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. epidermidis* (MRSE) and *Enterococcal* species, including *Enterococcus faecium* and *E. fecalis*, which express high-level of resistance to aminoglycosides and/or to vancomycin (VRE) [3]. These species are rapidly becoming multi-drug- or even pan-drug-resistant, thus tolerating most of the life-saving drugs. On this scenario, which urgently requires new antimicrobial solutions, natural antimicrobial cati-

onic peptides (AMPs) [3-6] represent effective antimicrobial agents, active on a wide variety of Gram-positive and Gram-negative bacteria, fungi, protozoa, and yeasts [8-11]. Due to their cationic structure, AMPs interact electrostatically with bacterial surfaces, diffuse inside the bacterial wall, and cause detrimental and irreversible alterations in the integrity of the membranes, such as pore formation, increasing permeabilization and leading to the loss of bacterial cytoplasmatic content and cell death [8-11]. Despite their efficacy, rapid action, and a low incidence in developing resistance [12], the widespread use of AMPs is hampered by their low biocompatibility, high instability, and high costs [8]. In recent years, less toxic, more stable, and low-cost synthetic mimics of AMPs, including cationic peptides, positively charged polymers and dendrimers have been developed and successfully tested as antimicrobial agents [8,13]. To this end, we recently synthetized seven amino acids-modified cationic dendrimers [14-16] and two ammonium hydrochloride copolymers [17,18] and assessed their antibacterial activity on numerous MDR Gram-positive and Gram-negative clinical isolates, obtaining very promising results [14-16,17,19]. The present work first reviews the synthesis and the physicochemical characteristics of the best cationic macromolecules recently developed by us, and then describes in detail their antibacterial effects and their rapid and not-lytic bactericidal behavior.

2. Synthesis of Cationic Macromolecules

The antibacterial cationic macromolecules recently reported by us include three small families of amino acids-modified biodegradable cationic dendrimers, and two types of polystyrene-based cationic random copolymers. In this paper, we reviewed the best representative of each category.

2.1. Synthesis of Amino Acids-Modified Cationic Dendrimers

2.1.1. Synthesis of the Fifth Generation (G5) Dendrimer Modified with 96 Peripheral Lysine (K) (G5K)

Dendrimer G5K was prepared according to reported procedures [20,21], by esterifying the tri-hydroxyl *core* 2,2-*bis*-hydroxymethylpropanol (*b*-HMP) with three equivalents of the acetonide protected G4-dendron-COOH (G4-A-D-COOH) made of repeated units of the AB_2 monomer - 2,2-*bis*-hydroxyethyl propanoic acids (*b*-HMPA) - and having 16 peripheral hydroxyl groups acetonide protected for each molecule. After deprotection of the peripheral acetonide groups, the G4-dendrimer having 48 peripheral hydroxyls was achieved, which was subsequently esterified with 48 equivalents of a lysine-modified G1-dendron producing G5K having 192 protonated nitrogen atoms [Scheme S1, Section S1, Supporting Information (SI)].

2.1.2. Synthesis of the Fifth Generation (G5) Dendrimer Peripherally Modified with 64 Lysine (K) (G5-PDK)

Dendrimer G5-PDK was prepared as previously reported [16], by esterifying the di-hydroxyl *core* 1,3-propandiol (PD) with two equivalents of the acetonide protected G5-dendron-COOH (G5-A-D-COOH) made of repeated units of the AB_2 monomer *b*-HMPA, and having 32 peripheral hydroxyl groups acetonide protected, each molecule. After deprotection of the peripheral acetonide groups, the obtained hydroxyl dendrimer having 64 OH groups, was subsequently esterified with 64 equivalents of *L*-lysine producing G5-PDK possessing 64 peripheral lysine and a total of 128 protonated nitrogen atoms (Scheme S2, Section S1, SI).

2.2. Synthesis of the Polystyrene-Based Cationic Random Copolymer P7

Copolymer P7 was prepared according to procedures previously reported [18], by free radical copolymerization in solution of the cationic monomer 2-methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride M7, with the comonomer *N*,*N*-di-methyl-acrylamide (DMAA) in methanol (MeOH) at 60°C (72 h), using azo-*bis*-isobutyronitrile

(AIBN) as radical initiator, and achieving a conversion of 85% (Scheme S3, Section S1, SI). M7 was in turn prepared by a multistep synthesis starting from the commercial methoxyacetic acid (Sigma-Aldrich, Darmstadt, Germany) [18].

3. Main Physicochemical Properties and Cytotoxicity Data of G5K, G5-PDK and P7

The main physicochemical properties of G5K, G5-PDK and of P7 have been included in Table S1 (Section S2, SI). A detailed discussion concerning these properties is available in the previously reported articles [14,16,18].

4. Antibacterial Activity of Cationic Macromolecules Reviewed in This Study

The minimal inhibitory concentrations values (MICs) for G5K, G5-PDK and P7 were obtained analyzing a total of 36, 18 and 61 strains of clinical origin, respectively. Although in several studies as that reported by Stenström *et al.* [22], MICs of 100 μM were considered as acceptable to establish significant antibacterial activity, in our opinion MICs > 512 μg/mL, corresponding to 16.5 μM (G5K), 25.4 μM (G5-PDK) and 37.3 μM (P7) were considered already too high to classify a compound as active. Accordingly, G5K was considered inactive against Gram-positive isolates and Gram-negative Enterobacteriaceae (MIC > 32.9 μM, Table S2, Section S3, SI).

Interestingly, G5K manifested consistent inhibitory activities against non-fermenting Gram-negative pathogens, including *P. aeruginosa, S. maltophylia,* and *A. baumannii* (Table S2). Against *P. aeruginosa* the activity of G5K was only slightly lower than that shown by the reported peptide dendrimer G3KL but was 3.6-fold higher than that of bH1 [23].

The antimicrobial activity of G5K against *A. baumannii* was comparable to that of G3KL and was 3.2–6-fold higher than that of bH1 [23]. Moreover, a MDR strain of *P. aeruginosa*, refractory even to ceftazidime/avibactam, was susceptible to the inhibitory activity of G5K, with a MIC of 2.07 μM. According to the MIC of G5K observed on *P. aeruginosa* (2.1 μM), G5K seemed slightly less powerful than colistin, whose sensitivity breck-point, identified by EUCAST, corresponds to 2μg/mL (1.59 μM) [14]. Among the *Pseudomonas* genus, other species, such as *P. putida*, *P. fluorescens, P. straminia* that can behave as opportunistic pathogens of humans and are responsible for severe infections, appeared to be even more susceptible to G5K (Table S2). The antimicrobial activity of G5K against all strains of *P. aeruginosa* essayed was 6.5-fold higher than that showed by the most active peptide dendrimer synthetized previously by Niederhafner *et al*. and tested against a not characterized strain of *P. aeruginosa (*MICs = 13.8 μM) [24]. The high antimicrobial activity of G5K, associated to its low toxicity against mammalian cells [14,20], may be ascribed to the presence of *L*-lysine (K), agreeing to previous reported findings [8,25-27].

The antibacterial activity of G5-PDK was firstly screened using a total of 7 clinical isolates of different genera belonging to Gram-positive and Gram-negative species. Interestingly, we detected remarkable effects specifically targeted toward the *Acinetobacter* genus [16]. Considering the clinical relevance of *A. baumannii* we have studied in more detail the antibacterial activity of G5-PDK on *Acinetobacter* determining the MIC values for 12 clinical isolates, including 6 *A. baumannii*, 2 *A. pittii*, one *A. johnsonii,* one *A. junii,* and two *A. ursingii* [16].

As observable in Table S2, G5-PDK was active against all the strains, displaying MICs of 3.2-12.7 μM. The studies on cationic materials tested in the last years against *A. baumannii* are limited, focusing mainly on AMPs, and the data reported are frequently conflicting, even when the same AMP was essayed on the same ATCC 19606 *A. baumannii* [28,29]. Referring to the more recent study of Vila-Farres *et al.* [29], with regard of MDR strains of *A. baumannii* (susceptible to colistin), G5-PDK was much more active than several AMPs which were tested on *Acinetobacter* ATCC.

G5-PDK was 3.4-6.8 times more active than Bactenecin, 4.7-9.4 times than Bufarin 1, over 6.6- times more effective than Histatin 5, 1.6-3.2-fold than Histatin 8, 1.2-2.4-fold than HNP-1 and 2, 2.1-4.2 times than Magainin 1 and even 8.2-16.4-fold than Magainin 2. β-Defensin, which, in the same experiment displayed MICs = 65.6 μM, was 5.2-10.4-fold less

active than G5-PDK, which in turn proved antibacterial effects comparable to those of Cecropin A, B, and Indolicidin [16,29]. In addition, G5-PDK showed MICs lower than those of Indolicin by 1.3-2.6 times and slightly higher than those of Mastoparam (6.3 μM *vs* 5.4 μM), when these AMPs were tested by Vila-Farres' group on clinical isolates of *A. baumannii*, as in our study [16,29]. G5-PDK was 1.4-2.8-fold more potent than Omiganan and 7.2-14.4-fold more powerful than Temporin A, considered in a study of Jaśkiewicz *et al.* [30].

Moreover, G5-PDK was 15.3-30.6-fold more potent than the synthetic all-D-enantiomer antimicrobial peptidomimetic, [D(KLAKLAK)$_2$], prepared by McGrath *et al.* to limit proteolysis which is the most concern associated to the *in vivo* use of AMPs [31].

G5-PDK was 2.6-5.1-fold more active than a cationic peptide (SA4) and even 5.2-10.4-fold than a cationic peptoid (SPO), recently prepared by the group of Sharma and tested on *A. baumannii* ATCC 19606 and on four MDR *A. baumannii* isolates [32].

Among the limited existing studies on cationic dendrimers against clinical isolates of *A. baumannii*, the most interesting research concerns the well-known, and in the years optimized, synthetic dendrimer peptide G3KL proposed by João Pires and co-worker, which showed, against this specie, very low MICs (0.8-3.2 μM) [33].

However, as far as our knowledge on synthetic cationic dendrimers with antibacterial activity on *Acinetobacter* is concerned, G5-PDK is the one showing the MICs closest to the MIC of G3KL [16]. Note that we have also considered other species of the genus Acinetobacter, such as *A. pittii, A. johnsonii, A. junii* and *A. ursingii,* which could be pathogenic to humans and develop multidrug resistance, causing severe and difficult to resolve infections. Against such species, however, G5-PDK showed MICs even lower than those shown against *A. baumanni*. Unfortunately, it is so far impossible to make comparisons between the antibacterial activity of G5-PDK and that of other agents against these isolates, because, to the best of our knowledge, there is currently no study in which such isolates have been tested [15].

As for P7, the 61 strains herein reported were MDR isolates of both Gram-positive and Gram-negative species and included Gram-negative strains with modifications in the outer membrane, usually non-susceptible to cationic agents due to a reduced negative surface charge [34-38], (Table S2). The lowest MICs were observed against VRE *E. faecium* (0.6–1.15 μM), VRE *E. faecalis* (2.3 μM) and *Bacillus subtilis* (1.15 μM), while MICs in the range 0.6–4.6 μM, were observed against the methicillin-resistant (MRS) *Staphylococci*. Against MRSA isolates, P7 was 1.6-fold more active than the ACP1Gly molecule prepared by Barman *et al.*, and up to 12-fold more active when tested against *E. faecium* [39].

Compared to the homopolymer (Poly1) described by Gelman et al., P7 showed MICs of 4.3 and 4.3-8.3-fold lower against *B. subtilis* and *E. faecium*, respectively, and 2 times lower against *S. aureus* [40]. P7 proved to be far more potent than three random cationic copolymers (PAI1–PAI3) against MRSA, being their MICs = 14.9 μM (PAI2), 17.7 μM (PAI3) and 267.8 μM (PAI1) [41]. Against *S. epidermidis*, P7 was slightly less effective than PAI2, equally active to PAI3 and more potent than PAI1. Against the Gram-negative species tested, P7 displayed significant activity, particularly against *Yersinia enterocolitica* and *Providencia stuartii* (MIC = 9.3 μM)), known to be bacterial strains with modified membrane charges. P7 showed low MICs against all strains of *Klebsiella* (4.6–9.3 μM), *Pseudomonas* (2.3–9.3 μM), *E. coli* (2.3–4.6 μM), *Acinetobacter* (2.3–9.3 μM), and *Stenotrophomonas maltophylia* (2.3–9.3 μM) considered, as well as against *Salmonella* gr. B (4.6 μM).

Particularly, the MICs observed for P7 on *E. coli* were lower than those of the antimicrobial copolymers (P4, P6, P7, and P9) prepared by Mizutani *et al.* [42]. On *E. coli*, P7 was 5–10 and 44–88-fold more active also than CNPS-4 and CNSP-3 prepared by Wen *et al.* respectively and was over 10 and 35 times more active when tested against *S. aureus* [43]. Furthermore, on *E. coli*, P7 showed MICs 2–4 times lower than those of Poly1 mentioned above. Additionally, P7 was found more active than a derivative of the potent natural cationic peptide magainin II, known as Ala^{8,13,18}-magainin 2 amide, which has been reported to exhibit potent antimicrobial activity. Against some isolates of KPC-producing

K. pneumonia, P7 was more active than ACP1Gly [39]. Again, on *K. pneumoniae*, P7 (MICs = 4.6–9.3 μM), was less active than **2a**, but much more active than **2b**, **2a** and **2b** being two types of polyionenes prepared by Weiyang [44]. P7 was slightly less active than **2a** on *E. coli* and significantly less active on MRSA, but more active against *A. baumannii* and *P. aeruginosa*, while if compared to **2b**, P7 was much more powerful against all these species. Table S2 shows a comparison between the MICs observed for the cationic macromolecules examined in this study and the MICs obtained, on the same strains, for commonly used antibiotics. These data show that the cationic macromolecules developed by us can be considered efficient antibacterial agents, capable of inhibiting numerous MDR pathogens, against which many of the current antibiotics are no longer active.

5. Time–Kill Curves

Time–kill experiments were carried out using G5K, G5-PDK and P7 at concentrations four times the MICs on three strains of *P. aeruginosa*, two strains of *A. baumannii*, and one of *S. maltophylia* for G5K, on two strains of *A. baumanni,* one of *A. pittii,* and one of *A. ursingii* for G5-PDK and on three isolates of *P. aeruginosa*, two of *K. pneumoniae*, and three of *S. aureus* for P7. Figure S1, S2 and S3 (Section S4, SI), show the most representative curves obtained for G5K, G5-PDK and P7, respectively. Accordingly, G5K possessed a very strong bactericidal effect against *P. aeruginosa*, since a rapid decrease of > 4 logs in the original cell number was evident after one hour of exposure and was maintained for at least 6 h after incubation. Also, G5K proved to be bactericidal against *A. baumannii*, while the inhibition was less pronounced against *S. matophilia*. G5-PDK possessed an extremely strong bactericidal effect against all the tested isolates of *Acinetobacter* genus, and a reduction of 5 logs in the original cell number was observable after 2h of exposure to G5-PDK, for *A. baumannii* (strain 245, curve reported in Figure S2). Regrowth started for both G5K and G5-PDK after 6h of incubation for all the species tested. This behavior is like that already observed for cationic bactericidal peptides that kill on contact, such as colistin, where the initial killing is rapid, being produced as soon as 5 min after exposure to the antibiotic and is followed by regrowth after 24 h [45]. Interestingly, G5-PDK has shown a bactericidal activity 7.7-fold higher than that of the cationic peptide reported by McGratha *et al*, which was bactericidal followed by regrowth as G5-PDK, at extremely high concentration (194.6 μM) [31]. Overall, these results demonstrated a rapid bactericidal nature of G5-PDK against MDR *A. baumannii*. As for P7, it possessed a very strong bactericidal effect on all the assayed pathogens. A rapid decrease in the original cell number was evident already after 30 min of exposure to P7, with a total decrease in the original cell number after two hours, regardless of the bacterial species tested. In the subsequent period up to 24 h, no further regrowth was observed (Figure S3). To the best of our knowledge, cases in which the bactericidal behaviours of cationic materials last up to 24 h are rarely reported in the literature.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Scheme S1. Synthetic path to obtain G5K; Scheme S2. Synthetic path to obtain G5-PDK; Scheme S3. Synthetic path to obtain P7 by free radical copolymerization in solution; Table S1. Main physicochemical features of G5K, G5-PDK and of P7; Table S2. MICs of G5K, G5-PDK and P7 against the multi drug resistant bacteria tested in our studies compared, when possible, to the MICs of available antibiotics commonly used against the same species obtained by their antibiograms. MIC values were obtained from experiments carried out in triplicate1 and were expressed as μM concentrations. Numbers in round brackets indicate the numerosity of different strains tested for that species; Figure S1. Time-killing curves performed with G5K (at concentrations equal to 4 × MIC) on *P. aeruginosa*, *S. maltophilia*, and *A. baumannii*.; Figure S2. Time-killing curves performed with G5-PDK (at concentrations equal to 4 x MIC) on *A. baumannii* 245 and *A. baumannii* 279, protracted up to 24 h; Figure S3. Time–kill curves performed with P7 (at concentrations equal to 4 × MIC) on *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*.

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References

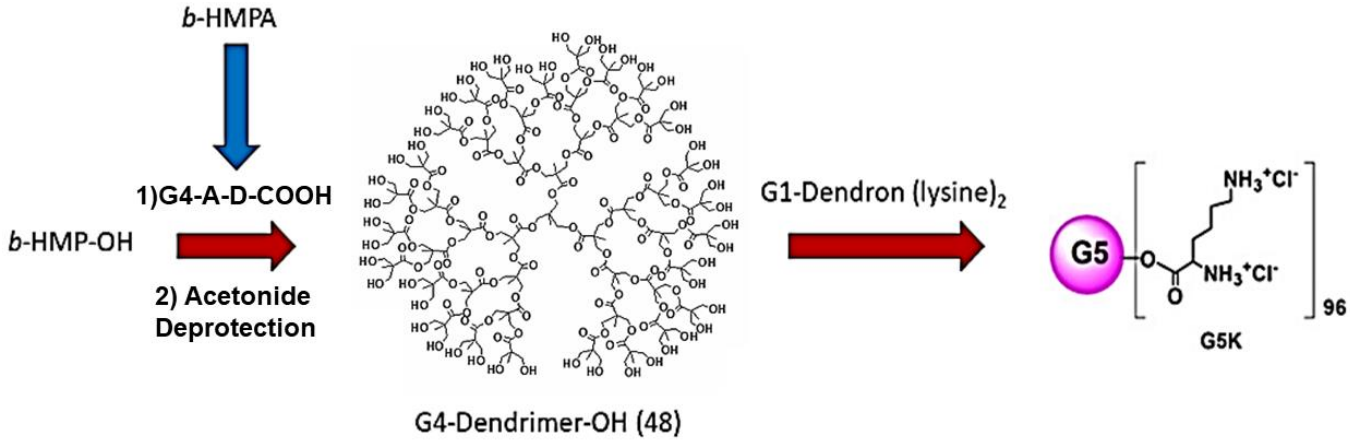
1. World Health Organization (WHO). *Prioritization of Pathogens to Guide Discovery, Research and Development of New Antibiotics for Drug Resistant Bacterial Infections, Including Tuberculosis*; WHO: Geneva, Switzerland, 2017; Available online: **https://www.who.int/medicines/areas/rational_use/PPLreport_2017_09_19.pdf?ua=1** (accessed on 02 July 2021).
2. World Health Organization (WHO). *No Time to Wait: Securing the Future from Drug-Resistant Infections. Report to the Secretary-General of the United Nations. Interagency Coordination Group on Antimicrobial Resistance*; WHO: Geneva, Switzerland, 2019; Available online: **https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG_final_report_EN.pdf?ua=1** (accessed on 02 July 2021).
3. Koulenti, D.; Xu, E.; Mok, I.Y.S.; Song, A.; Karageorgopoulos, D.E.; Armaganidis, A.; Lipman, J.; Tsiodras, S. Novel Antibiotics for Multidrug-Resistant Gram-Positive Microorganisms. *Microorganisms* **2019**, *7*, 270.
4. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389–395.
5. Seo, M.D.; Won, H.S.; Kim, J.H.; Mishig-Ochir, T.; Lee, B.J. Antimicrobial peptides for therapeutic applications: A review. *Molecules* **2012**, *17*, 12276–12286.
6. Gordon, Y.J.; Romanowski, E.G.; McDermott, A.M. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res.* **2005**, *30*, 505–515.
7. Landman, D.; Georgescu, C.; Martin, D.A.; Quale, J. Polymyxins revisited. *Clin. Microbiol. Rev.* **2008**, *21*, 449–465.
8. Alfei, S.; Schito, A. Positively charged polymers as promising devices against multidrug resistant Gram-Negative bacteria: A review. *Polymers* **2020**, *12*, e1195.
9. Kang, H.K.; Kim, C.; Seo, C.H.; Park, Y. The therapeutic applications of antimicrobial peptides (AMPs): A patent review. *J. Microbiol.* **2017**, *55*, 1–12.
10. Brogden, K.A. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **2005**, *3*, 238–250.
11. Yang, L.; Gordon, V.D.; Trinkle, D.; Schmidt, N.W.; Davis, M.A.; Devries, C.; Som, A.; Cronan, J.E.; Tew, G.N.; Wong, G.C.L. Mechanism of a prototypical synthetic membrane-active antimicrobial: Efficient hole-punching via interaction with negative intrinsic curvature lipids. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 20595–20600.
12. Spohn, R.; Daruka, L.; Lázár, V.; Martins, A.; Vidovics, F.; Grézal, G.; Méhi, O.; Kintses, B.; Számel, M.; Jangir, P.K.; et al. Integrated evolutionary analysis reveals antimicrobial peptides with limited resistance. *Nat. Commun.* **2019**, *10*, e4538.
13. Alfei, S.; Schito, A.M. From Nanobiotechnology, Positively Charged Biomimetic Dendrimers as Novel Antibacterial Agents: A Review. *Nanomaterials* **2020**, *10*, 2022. https://doi.org/10.3390/nano10102022
14. Schito, A.M.; Alfei, S. Antibacterial Activity of Non-Cytotoxic, Amino Acid-Modified Polycationic Dendrimers against *Pseudomonas aeruginosa* and Other Non-Fermenting Gram-Negative Bacteria. *Polymers* **2020**, *12*, 1818. https://doi.org/10.3390/polym12081818.
15. Schito, A.M.; Schito, G.C.; Alfei, S. Synthesis and Antibacterial Activity of Cationic Amino Acid-Conjugated Dendrimers Loaded with a Mixture of Two Triterpenoid Acids. *Polymers* **2021**, *13*, 521. https://doi.org/10.3390/polym13040521.
16. Alfei, S.; Caviglia, D.; Piatti, G.; Zuccari, G.; Schito, A.M. Bactericidal Activity of a Self-Biodegradable Lysine-Containing Dendrimer against Clinical Isolates of Acinetobacter Genus. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22147274.
17. Alfei, S.; Piatti, G.; Caviglia, D.; Schito, A.M. Synthesis, Characterization, and Bactericidal Activity of a 4-Ammoniumbuthylstyrene-Based Random Copolymer. *Polymers* **2021**, *13*, 1140. https://doi.org/10.3390/polym13071140.

18. Alfei, S.; Marengo, B.; Valenti, G.E.; Domenicotti, C. Synthesis of Polystyrene-Based Cationic Nanomaterials with Pro-Oxidant Cytotoxic Activity on Etoposide-Resistant Neuroblastoma Cells. *Nanomaterials* **2021**, *11*, 977. https://doi.org/10.3390/nano11040977.
19. Schito, A.M.; Piatti, G.; Caviglia, D.; Zuccari, G.; Alfei, S. Broad-Spectrum Bactericidal Activity of a Synthetic Random Copolymer Based on 2-Methoxy-6-(4-Vinylbenzyloxy)-Benzylammonium Hydrochloride. *Int. J. Mol. Sci.* **2021**, *22*, 5021. https://doi.org/10.3390/ijms22095021.
20. Alfei, S.; Castellaro, S.; Taptue, G.B. Synthesis and NMR characterization of dendrimers based on 2, 2-*bis*-(hydroxymethyl)-propanoic acid (*bis*-HMPA) containing peripheral amino acid residues for gene transfection. *Org. Commun.* **2017**, *10*, 144–177.
21. Alfei, S.; Castellaro, S. Synthesis and characterization of polyester-based dendrimers containing peripheral arginine or mixed amino acids as potential vectors for gene and drug delivery. *Macromol. Res.* **2017**, *25*, 1172–1186.
22. Stenström, P.; Hjorth, E.; Zhang, Y.; Andrén, O.C.J.; Guette-Marquet, S.; Schultzberg, M.; Malkoch, M. Synthesis and in vitro evaluation of monodisperse amino-functional polyester dendrimers with rapid degradability and antibacterial properties. *Biomacromolecules* **2017**, *18*, 4323–4330.
23. Stach, M.; Siriwardena, T.N.; Köhler, T.; Van Delden, C.; Darbre, T.; Reymond, J.-L. Combining topology and sequence design for the discovery of potent antimicrobial peptide dendrimers against multidrug-resistant pseudomonas aeruginosa. *Angew. Chem. Int. Ed.* **2014**, *53*, 12827–12831.
24. Niederhafner, P.; Bednárová, L.; Buděšínský, M.; Šafařík, M.; Ehala, S.; Ježek, J.; Borovičková, L.; Fučík, V.; Čeřovský, V.; Slaninová, J. Melectin MAPs: The influence of dendrimerization on antimicrobial and hemolytic activity. *Amino Acids* **2010**, *39*, 1553–1561.
25. Glukhov, E.; Stark, M.; Burrows, L.L.; Deber, C.M. Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. *J. Biol. Chem.* **2005**, *280*, 33960–33967.
26. Ganewatta, M.S.; Tang, C. Controlling macromolecular structures towards effective antimicrobial polymers. *Polymer* **2015**, *63*, A1–A29.
27. Yang, X.; Hu, K.; Hu, G.; Shi, D.; Jiang, Y.; Hui, L.; Zhu, R.; Xie, Y.; Yang, L. Long hydrophilic-and-cationic polymers: A different pathway toward preferential activity against bacterial over mammalian membranes. *Biomacromol.* **2014**, *15*, 3267–3277.
28. Giacometti A, Cirioni O, Del Prete MS, Barchiesi F, Paggi AM, Petrelli E, Scalise G. Comparative activities of polycationic peptides and clinically used antimicrobial agents against multidrug-resistant nosocomial isolates of Acinetobacter baumannii. *J. Antimicrob. Chemother*, **2000**, *46*, 807–810. http://dx.doi.org/10.1093/jac/46.5.807.
29. Vila-Farres, X.; Garcia de la Maria, C.; Lopez-Rojas, R.; Pachon, J.; Giralt, E.; Vila, J. In vitro activity of several antimicrobial peptides against colistinsusceptible and colistin-resistant Acinetobacter baumannii. *Clin. Microbiol. Infect.* **2012**, *18*, 383–387. 10.1111/j.1469-0691.2011.03581.x.
30. Jaśkiewicz, M.; Neubauer, D.; Kazor, K. *et al.* Antimicrobial Activity of Selected Antimicrobial Peptides Against Planktonic Culture and Biofilm of *Acinetobacter baumannii*. *Probiotics & Antimicro. Prot.* **2019**, *11*, 317–324 (2019). https://doi.org/10.1007/s12602-018-9444-5
31. McGratha, D.M; Barbua, E.M; Driessena, WHP.; Lascob, TM.; Tarrand, JJ.; Okhuysend, PC.; Kontoyiannise, DP.; Sidmanf, RL.; Pasqualinia, P.; Arapa, W. Mechanism of action and initial evaluation of a membrane active all-D-enantiomer antimicrobial peptidomimetic. *PNAS*, **2013**, *110*, 3477-3482.
32. D. Sharma et al. *Biochem. Biophys. Res. Commun.* **2019**, *518*, e478475.
33. Pires, J.; Siriwardena, T.N.; Stach, M.; Tinguely, R.; Kasraian, S.; Luzzaro, F.; Leib, S.L.; Darbre, T.; Reymond, J.-L.; Endimiani, A. *In vitro* activity of the novel antimicrobial peptide dendrimer G3KL against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **2015**, *59*, 7915–7918. doi:10.1128/AAC.01853.
34. Lee, J.-Y.; Park, Y.K.; Chung, E.S.; Na, I.Y.; Ko, K.S. Evolved Resistance to Colistin and Its Loss Due to Genetic Reversion in Pseudomonas Aeruginosa. *Sci. Rep.* **2016**, *6*, 25543.
35. Pacheco, T.; Bustos, R.-H.; González, D.; Garzón, V.; García, J.-C.; Ramírez, D. An Approach to Measuring Colistin Plasma Levels Regarding the Treatment of Multidrug-Resistant Bacterial Infection. *Antibiotics* **2019**, *8*, 100.
36. Liu, H.; Zhu, J.; Hu, Q.; Rao, X. Morganella morganii, a non-negligent opportunistic pathogen. *Int. J. Infect. Dis.* **2016**, *50*, 10–17.
37. Band, V.I.; Weiss, D.S. Mechanisms of Antimicrobial Peptide Resistance in Gram-Negative Bacteria. *Antibiotics* **2015**, *4*, 18–41.
38. Ostrowska, K.; Kamysz, W.; Dawgul, M.; Różalski, A. Synthetic amphibian peptides and short amino-acids derivatives against planktonic cells and mature biofilm of Providencia stuartii clinical strains. *Pol. J. Microbiol.* **2014**, *63*, 423–431.
39. Barman, S.; Konai, M.M.; Samaddar, S.; Haldar, J. Amino Acid Conjugated Polymers: Antibacterial Agents Effective against Drug-Resistant Acinetobacter baumannii with No Detectable Resistance. *Acs Appl. Mater. Interfaces* **2019**, *11*, 33559–33572.
40. Gelman, M.A.; Weisblum, B.; Lynn, D.M.; Gellman, S.H. Biocidal activity of polystyrenes that are cationic by virtue of protonation. *Org. Lett.* **2004**, *6*, 557–560.
41. Locock, K.E.S.; Michl, T.D.; Stevens, N.; Hayball, J.D.; Vasilev, K.; Postma, A.; Griesser, H.J.; Meagher, L.; Haeussler, M. Antimicrobial Polymethacrylates Synthesized as Mimics of Tryptophan-Rich Cationic Peptides. *ACS Macro Lett.* **2014**, *3*, 319–323.
42. Mizutani, M.; Palermo, E.F.; Thoma, L.M.; Satoh, K.; Kamigaito, M.; Kuroda, K. Design and Synthesis of Self-Degradable Antibacterial Polymers by Simultaneous Chain- and Step-Growth Radical Copolymerization. *Biomacromolecules* **2012**, *13*, 1554–1563.
43. Wen, Q.; Xu, L.; Xiao, X.; Wang, Z. Preparation, characterization, and antibacterial activity of cationic nanopolystyrenes. *J. Appl. Polym. Sci.* **2019**, *137*, 48405.

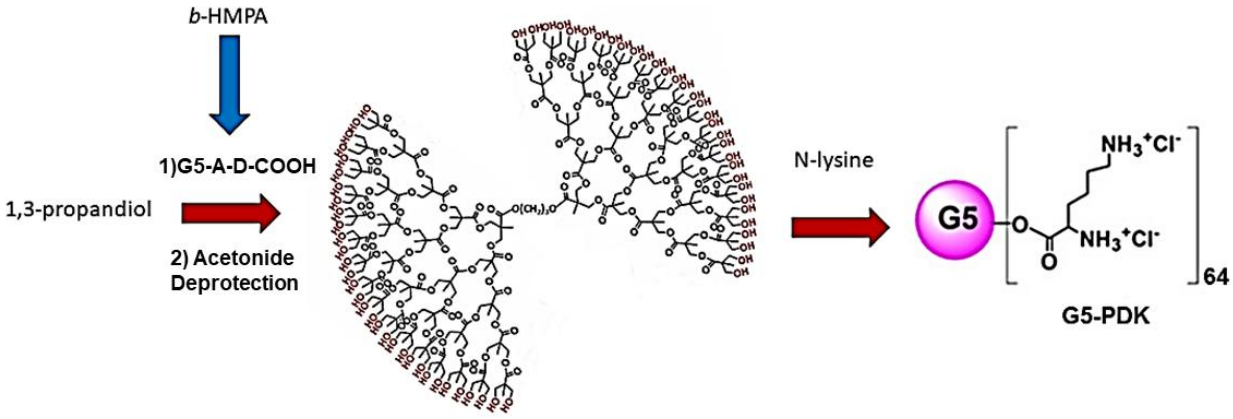
44. Weiyang, L.; Shrinivas, V.; Guansheng, Z.; Bisha, D.; Tan, P.K.J.; Xu, L.; Weimin, F.; Yi, Y.Y. Antimicrobial polymers as therapeutics for treatment of multidrug-resistant Klebsiella pneumoniae lung infection. *Acta Biomater.* **2018**, *78*, 78–88.
45. Landman, D.; Georgescu, C.; Martin, D.A.; Quale, J. Polymyxins revisited. *Clin. Microbiol. Rev.* **2008**, *21*, 449–465.

Supplementary Information

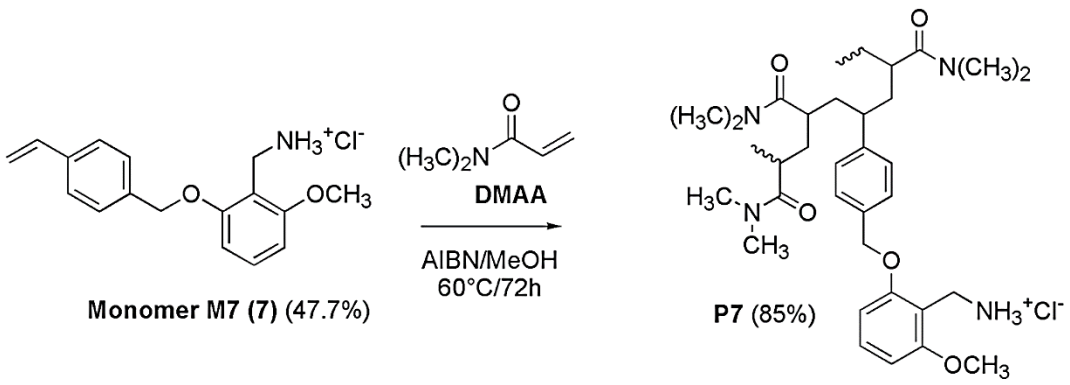
Section S1. Synthesis of Cationic Macromolecules



Scheme 1. Synthetic path to obtain G5K.



Scheme 2. Synthetic path to obtain G5-PDK.



Scheme 3. Synthetic path to obtain P7 by free radical copolymerization in solution.

Section S2. Main Physicochemical Properties and Cytotoxicity Data of the Reviewed Cationic Macromolecules

Table S1. Main physicochemical features of G5K, G5-PDK and of P7.

Entry	Analysis	Feature	Determinations	
G5K * 192 HCl	Elemental Analysis	C, H, N, Cl	41.88, 7.35, 9.15, 22.17* 41.57, 7.00, 8.90, 22.52§	
	^{1}H NMR	Molecular Weight (MW)§	30224	
	Volumetric titration	Molecular Weight (MW)*	28966 (error:-4.2%)	
	Cytotoxicity to CHO cells (200 μg/mL; 6.6 μM)	Cells viability (%)	97.2	
	Potentiometric Titration	Max dpH/dV [1]	7.80	7.65
		HCl 0.1N (mL) [2]	0.8	1.8
		pH [3]	7.32	3.33
G5PDK * 128 HCl	Elemental Analysis	C, H, N, Cl	41.78, 7.30, 9.10, 22.11* 41.56, 7.00, 8.90, 22.53§	
	^{1}H NMR	Molecular Weight (MW)§	20145	
	Volumetric titration	Molecular Weight (MW)*	19961 (error:-0.9%)	
	Cytotoxicity to HaCaT# (503.6 μg/mL; 25 μM; 24h)	Cells viability (%) SI (24h)	95.0 13-404	
	DLS [4] Analysis	Z-Ave [5] (nm)	203.0±2.6	
		PDI [6]	0.282±0.028	
		Z-potential [7] (ζ-p)	+19.2±7.3	
	Potentiometric Titration	Max dpH/dV [1]	10.75	4.0
		HCl 0.1N (mL) [2]	0.6	1.2
		pH [3]	6.85	4.80
P7	VPO [8] analysis (MeOH, 45°C)	Average Molecular Mass (Mn)	13719	
	Volumetric Titration	μequivNH_2/gP7	305	
	Cytotoxicity to HaCaT# (13.7 μg/mL; 1 μM; 24h)	Cells viability (%) SI (24h)	90.4 >1 (1.2-4.7)**	
	DLS [4] Analysis	Z-Ave [5] (nm)	220±18	
		PDI [6]	0.809±0.004	
		Z-potential [7] (ζ-p)	+49.8±5.8	
	Potentiometric Titration	Max dpH/dV [1]	10.75	4.0
		HCl 0.1N (mL) [2]	0.6	1.2
		pH [3]	6.85	4.80

G = generation; PD = propandiol; K = lysine; SI = selectivity index (DL_{50}/MICs); * found/observed; § required/computed; # human keratinocytes; ** towards *Enterococci, S. epidermidis,* minor *Staphylococci, B. subtilis,* some *E.coli, A. pittii, P. putida,* some *P. aeruginosa* and *S. maltophylia*; [1] max values of first derivative curve of titration curve, indicating the existance of a two-step protonation process; [2] volumes of HCl 0.1N needed to protonate the dendrimer/copolymer; [3] pH values at which protonations occur; [4] Dinamic Light Scatteing; [5] hydrodinamic diameter of particles; [6] polydispersivity index; [7] measure of the electrical charge of particles suspended in the liquid of acquisition (water); [8] vapor-pressure osmometry.

Section S3. Antibacterial Activity of the Cationic Macromolecules Reviewed in This Study

Table S2. MICs of G5K, G5-PDK and P7 against the multi drug resistant (MDR) bacteria tested in our studies compared, when possible, to the MICs of available antibiotics commonly used against the same species obtained by their antibiograms. MIC values were obtained from experiments carried out in triplicate[1] and were expressed as μM concentrations. Numbers in round brackets indicate the numerosity of different strains tested for that species.

	G5K (30224) [2]	**G5-PDK (20145)** [2]	**P7 (13719)**[2]	**Commercial Antibiotics**
Strains	**MIC** (μM)	**MIC** (μM)	**MIC** (μM)	**MIC** (μM)
Enterococcus genus				
*E. faecalis**	32.9 (2)	>25.4 (1)	2.3 (4)	22.1-88.3 [3]
*E. faecium**	32.9 (2)	>25.4 (1)	0.6-1.15 (3)	88.3-176.6 [3]
E. casseliflavus	n.t.	n.t.	1.15 (1)	
E. durans	n.t.	n.t.	1.15 (1)	
E. gallinarum	n.t.	n.t.	1.15 (1)	
Staphylococcus genus				
*S. aureus***	32.9 (2)	>25.4 (1)	4.6 (3)	637.7-1275 [4]
*S. epidermidis****	32.9 (2)	>25.4 (1)	1.15 (3)	637.7 [4]
S. haemolyticus 193 **	n.t.	n.t.	1.15 (1)	
S. hominis 125 **	n.t.	n.t.	0.6 (1)	
S. simulans 163 **	n.t.	n.t.	1.15 (1)	
Sporogenic isolate				
B. subtilis	n.t.	n.t.	1.15 (1)	212.4 [5]
Enterobacteriaceae family				
E. coli#,§	32.9 (2)	>25.4 (1)	2.3-4.6 (3)	16.8 [6] 96.6 [7] 13.3-26.5 [5]
K. pneumoniae#	32.9 (2)	>25.4 (1)	4.6-9.3 (7)	4.4-16.8 [6] 96.6-193.2 [7] 6.7-13.3 [5]
P. mirabilis	32.9 (1)	n.t.	18.6 (1)	
Y. enterocolitica	n.t.	n.t.	9.3 (1)	
S. marcescens	n.t.	n.t.	>18.6 (1)	
M. morganii	n.t.	n.t.	18.6 (1)	212.4 [5]
K. oxytoca	n.t.	n.t.	9.3 (1)	
Salmonella gr.B	n.t.	n.t.	4.6 (1)	235.5 [8]
P. stuartii	n.t.	n.t.	9.3 (1)	212.4 [5]
Non-fermenting species				
A. baumannii	2.1 (4)	6.3-12.7 (6)	2.3-9.3 (5)	1.6-193.2 [7]
A. pittii	2.1 (1)	6.3 (2)	2.3 (1)	1.6-3.2 [7]
A. johnsonii	n.t.	6.3 (1)	n.t.	0.9 [7]
A. junii	n.t.	12.7 (1)	n.t.	0.4 [7]
A. ursingii	n.t.	3.2-6.3 (2)	n.t.	0.4-0.8 [7]
P. aeruginosa	2.1 (11)	n.t.	2.3-9.3 (7)	19.6-156.5 [9]
P. fluorescens	0.5 (1)	n.t.	2.3 (1)	
P. putida	1.0 (1)	n.t.	4.6 (1)	
P. straminea	1.0 (1)	n.t.	n.t.	
S. maltophylia	2.1-4.2 (4)	n.t.	2.3-9.3 (6)	58.8-117.7 [8]

n.t. = not tested; [1] the degree of concordance was in all the experiments 3/3, and standard deviation (±SD) was zero; [2] MW of G5K and G5-PDK and Mn of copolymer P7; [3] vancomycin; [4] oxacillin; [5] amoxy-clavulanate; [6] ertapenem; [7] ciprofloxacin; [8] trimethoprim-sulfamethoxazole; [9] piperacillin tazobactam; * denotes vancomycin resistant (VRE); ** denotes methicillin resistant; *** denotes resistance toward methicillin and linezolid; # denotes carbapenemase (KPC)-producing; § denotes O157:H7; *P. aeruginosa, S. maltophylia* and *A. baumannii* are all MDR bacteria.

Section S4. Time-kill Experiments

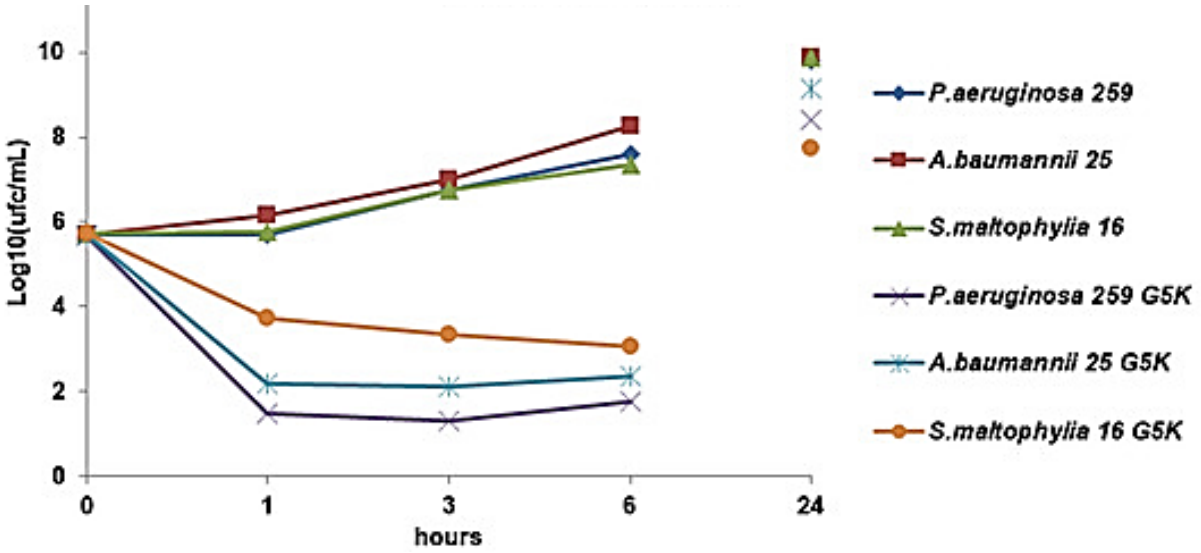


Figure S1. Time-kill curves performed with G5K (at concentrations equal to 4 × MIC) on *P. aeruginosa, S. maltophilia,* and *A. baumannii*.

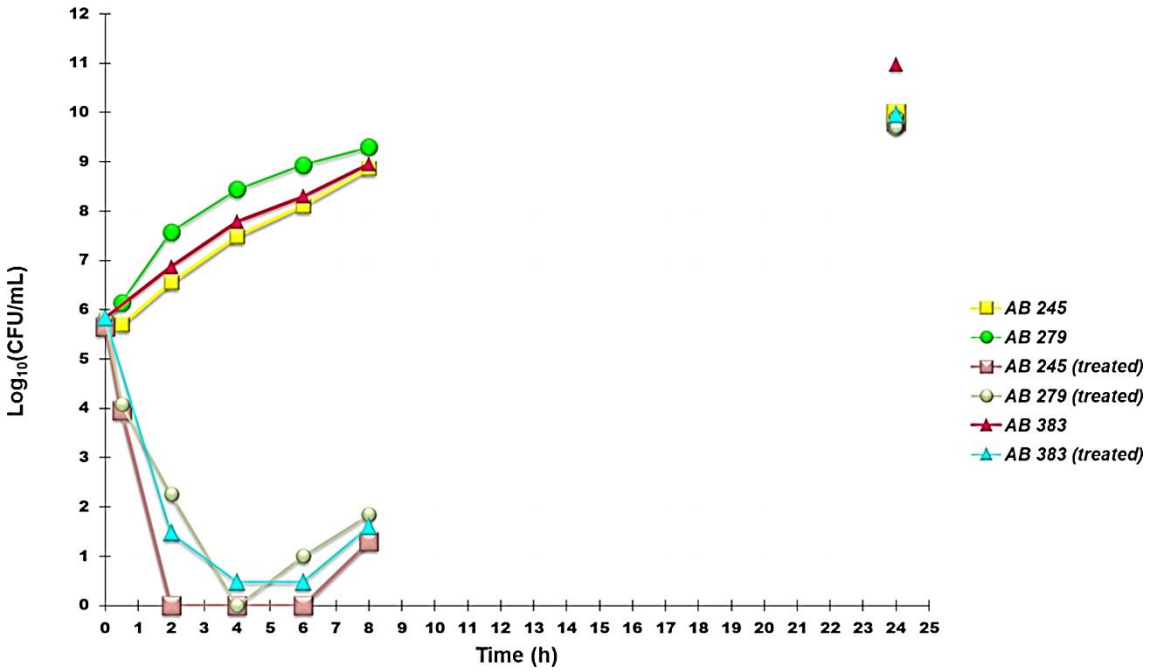


Figure S2. Time-kill curves performed with G5-PDK (at concentrations equal to 4 x MIC) on *A. baumannii* 245, A. baumannii 279 and *A. baumannii* 383.

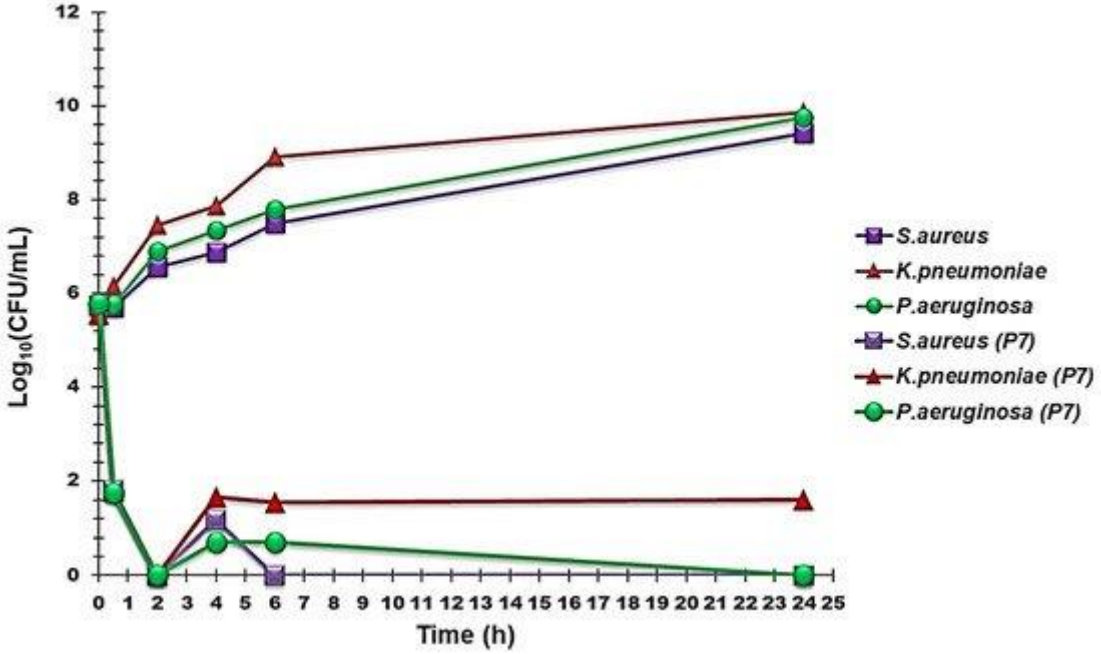


Figure S3. Time–kill curves performed with P7 (at concentrations equal to 4 × MIC) on *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*.