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

Silvana Alfei [1,*], Gabriella Piatti [2], Debora Caviglia [2], Gian Carlo Schito [2], Guendalina Zuccari [1] and Anna Maria Schito [2]

[1] Department of Pharmacy, University of Genoa, Viale Cembrano, 16148 Genoa, Italy; zuccari@difar.unige.it
[2] Department of Surgical Sciences and Integrated Diagnostics (DISC), University of Genoa, Viale Benedetto XV, 6, 16132 Genova, Italy; gabriella.piatti@unige.it (G.P.); cavigliad86@gmail.com (D.C.); giancarlo.schito@unige.it (G.C.S.); amschito@unige.it (A.M.S.)
* Correspondence: alfei@difar.unige.it; Tel.: +39-010-355-2296

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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 with pathogens' surfaces, 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 h-time-killing experiments, all of the mentioned macromolecules displayed rapid bactericidal effects, while when tested on human keratinocytes, especially G5-PDK, showed low levels of cytotoxicity and high values of selectivity indices. 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 of the bacterial resistance to current antibiotics.

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

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1. Introduction

Nowadays, the rapid and worldwide development of multi-drug resistant (MDR) bacteria, responsible for 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 of causing 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 maltophilia* 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 a high level of resistance to aminoglycosides and/or to vancomycin (VRE) [3]. These species are rapidly becoming multidrug- or even pan-drug-resistant, thus tolerating most of the life-saving drugs. In this scenario,

which urgently requires new antimicrobial solutions, natural antimicrobial cationic peptides (AMPs) [3–7] 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 to 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 of 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 [14–17,19]. 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 the 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 with 16 peripheral hydroxyl groups acetonide protected for each molecule. After deprotection of the peripheral acetonide groups, the G4-dendrimer with 48 peripheral hydroxyls was achieved, which was subsequently esterified with 48 equivalents of a lysine-modified G1-dendron-producing G5K with 192 protonated nitrogen atoms [Scheme S1, Section S1, Supplementary Materials].

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 for each molecule. After deprotection of the peripheral acetonide groups, the obtained hydroxyl dendrimer with 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, Supplementary Materials).

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

Copolymer P7 was prepared according to the procedures previously reported [18] by free-radical copolymerization in a solution of the cationic monomer 2-methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride M7, with the comonomer *N*,*N*-di-methylacrylamide (DMAA) in methanol (MeOH) at 60 °C (72 h), using azo-*bis*-isobutyronitrile (AIBN) as a radical initiator, and achieving a conversion of 85% (Scheme S3, Section S1,

Supplementary Materials). M7 was in turn prepared by a multistep synthesis starting from the commercial methoxy-acetic 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 P7 have been included in Table S1 (Section S2, Supplementary Materials). 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 concentration values (MICs) for G5K, G5-PDK, and P7 were obtained by 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 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, Supplementary Materials).

Interestingly, G5K manifested consistent inhibitory activities against non-fermenting Gram-negative pathogens, including *P. aeruginosa*, *S. maltophilia*, 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, an MDR strain of *P. aeruginosa*, refractory even to ceftazidime/avibactam, was susceptible to the inhibitory activity of G5K, with an 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 breakpoint, identified by EUCAST, corresponds to 2 μg/mL (1.59 μM) [14]. Among the *Pseudomonas* genus, other species, such as *P. putida*, *P. fluorescens*, and *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 shown by the most active peptide dendrimer synthetized previously by Niederhafner et al. and tested against a non-characterized strain of *P. aeruginosa* (MICs = 13.8 μM) [24]. The high antimicrobial activity of G5K, associated with its low toxicity against mammalian cells [14,20], may be ascribed to the presence of *L*-lysine (K), agreeing with previously 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 six *A. baumannii*, two *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 to 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 more than Bufarin 1, over 6.6 times more effective than Histatin 5, 1.6–3.2-fold that of Histatin 8, 1.2–2.4-fold that of HNP-1 and 2, 2.1–4.2 times more than Magainin 1, and even 8.2–16.4-fold that of 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 by 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 with 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 more 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 of 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 could 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 of 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, their MICs being = 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 maltophilia* (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 also 5–10 and 44–88-fold more active 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 to be 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. maltophilia* 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. Figures S1–S3 (Section S4, Supplementary Materials) 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 >four 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 the *Acinetobacter* genus, and a reduction of five logs in the original cell number was observable after 2 h of exposure to G5-PDK for *A. baumannii* (strain 245, curve reported in Figure S2). Regrowth started for both G5K and G5-PDK after 6 h 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 an 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 behaviors of cationic materials last up to 24 h are rarely reported in the literature.

6. Conclusions

For meeting the worldwide need for new antimicrobial compounds against multidrug-resistant (MDR) bacteria, we synthetized seven amino acids-modified cationic dendrimers and two ammonium hydrochloride copolymers and assessed their antibacterial activity on numerous MDR Gram-positive and Gram-negative clinical isolates, obtaining very promising results. Here the synthesis, the physicochemical characteristics, and the cytotoxic behavior of the best cationic macromolecules obtained, as well as their antibacterial effects and their rapid and not-lytic bactericidal activity, have been reviewed. Particularly, dendrimers G5K and G5-PDK, even if of different MW, were both of fifth generation, and were both in the form of nanosized cationic biodegradable nanoparticles (NPs) peripherally decorated with lysine, possessing high solubility in water. Although inactive against bacteria of Gram-positive species and *Enterobacteriaceae*, G5K NPs manifested consistent inhibitory activities against non-fermenting Gram-negative pathogens, such as *P. aeruginosa, S. maltophilia,* and *A. baumannii*, and rapid bactericidal effects especially against isolates of *P. aeruginosa* and *A. baumannii*. On the contrary, G5-PDK manifested consistent inhibitory

and bactericidal effects specifically against several isolates of the genus *Acinetobacter.* The selectivity indices (SIs) determined from results by cytotoxicity experiments carried out on human keratinocytes at 24 h were from high (4.5–38.2 for G5K) to extremely high (13–404 for G5-PDK) thus establishing their applicability in therapy to counteract infections sustained by the most frightening non-fermenting Gram-negative isolates and by *A. baumannii*, respectively. Copolymer P7 was similarly in the form of cationic NPs highly soluble in water. Although copolymer P7 demonstrated a higher level of cytotoxicity and selectivity indices significantly lower than those of dendrimers (SIs >1 on Gram-positive species and <1 on Gram-negative isolates), it proved to have very potent and broad-spectrum antibacterial and bactericidal effects, thus being suitable as an environmental disinfectant or for other non-clinical uses where a very effective device is needed to totally annihilate pathogens of various species despite their resistance to the antibiotics currently available. Considering the scenario presented in this paper, we thought it could be interesting to prepare the correspondent crosslinked insoluble resins of copolymer P7 and assess their antibacterial behavior, because, if functioning, they could represent an interesting new material usable to purify waters polluted by bacteria. The resins have been prepared and microbiologic investigations are ongoing with very promising results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ECMS2021-10833/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 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 × 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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Data Availability Statement: All data concerning this study are contained in the present manuscript or in previous articles whose references have been provided.

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