Antibacterial Cationic Peptide Dendrimers

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On the alarming scenario of the increasing antimicrobial resistance, causing a growing number of untreatable bacterial infections, we decided to report in a serie of entries, the state of the art concerning the development and application of the main types of cationic antibacterial dendrimers, proposed as unconventional options to the no longer effective traditional antibiotics. In a first recent entry, a general overview including an introduction to the topic, and sections which summarize the main types of dendrimers in existence and the main ones that have demonstrated antibacterial properties disclosed in the past decade, the main types of cationic antibacterial dendrimers have been introduced. In particular, the PAMAM and PPI-based cationic dendrimers developed in the last decade, which showed considerable antibacterial properties, have been reviewed. In this second entry, we have provided an updated overview concerning the most studied class of antibacterial cationic dendrimers, i.e. the antimicrobial peptide ones. Significant case studies concerning the most active agents belonging to this category which have been prepared in the last decade have been reported.

Keywords: bacterial resistance ; traditional ineffective antibiotics ; natural antimicrobial peptide ; antibacterial cationic dendrimers ; antibacterial antimicrobial dendrimer peptides

1. Introduction

The increasing growth of resistant bacterial strains has caused the appearance and the re-emergence of serious infections, in particular in nosocomial settings, difficult to be treated with traditional antibiotics. Furthermore, because bacterial infections, especially if caused by biofilm production, hinder the durability, reliability, and performance of many medical devices and implants, antibiofilm strategies such as antibacterial coatings that repel bacteria and prevent biofilm formation are highly desirable. In this regard, appealing molecules are represented by natural cationic antimicrobial peptides (CAMPs), which are capable to kill pathogens simply through external contact, without the need to address the numerous resistance mechanisms due to genetic mutations that bacteria can develop. Inspired by natural CAMPs, cationic antimicrobial macromolecules including dendrimers have shown to function as antibacterial agents and as antimicrobial surface coatings as well. Different types of Ds, including cationic ones, have been developed for the treatment of infections sustained by multidrug-resistant bacteria, mainly during the last decade. Commercially available PAMAM and PPI-based dendrimers, as well as several their dendrimer derivatives which proved to possess in vitro broad-spectrum activities, have been reviewed in a recent entry on Encyclopedia (https://encyclopedia.pub/7167). In this second entry, we have provided an updated overview concerning the most studied class of antibacterial cationic dendrimers, i.e. the antimicrobial peptide ones. Significant case studies concerning the most active agents belonging to this category which have been prepared in the last decade have been reported.

2. Cationic Peptides Dendrimers

Natural CAMPs such as polymyxins or gramicidins, known to display high antimicrobial potency unfortunately associate with remarkable cytotoxicity to the host cells, have inspired the synthesis of cationic antimicrobial peptides. In this regard, cationic peptides with repeating sequences of arginine and tryptophan, (RW)n, have highlighted that short chains of R and W comprise a pharmacophore for mimicking the antimicrobial activity of the natural CAMPs [1]. Studies have demonstrated that the presence of multivalent dipeptides or tetrapeptides on different scaffolds, further enhances the antibacterial effects. In this context, since multivalence is one of the nonpareil properties of Ds, six new Ds, on which different dipeptide combinations of cationic and hydrophobic amino acids are linked to a four-branched lysine dendritic *core* were constructed by Young et al. (2011) [1]. In addition to RW, these dipeptides include RF, RY, KW, KF, KY, and HW sequences. These materials were compared with the correspondent linear and polymeric materials for their antimicrobial activity against both Gram-negative *E. coli* and *A. baumannii* species, as well as towards the Gram-positive *S. aureus* and for their hemolytic toxicity. Interestingly, more than one dendrimer peptide proved to possess antimicrobial activity higher than that of both linear and polymeric correspondent peptides and lower hemolytic toxicity. In particular, the better performant dendrimer peptide, named (RW)4D, provided IC_{50} values (concentration of the agent that

inhibit 50% of bacterial growth) of 3.9, 15 and 42 μg/mL against *E. coli*, *A. Baumannii* and *S. aureus* respectively, associated with a very low hemolytic toxicity, since the hemolytic cytotoxicity (HC_{50}) value (concentration of agent that lyses 50% of RBC) was 1962 μg/mL. In addition, the results of the study established that even extended exposure to sub-lethal doses of (RW)4D elicited much lower levels of resistance than traditional antibiotics or antimicrobials such as ciprofloxacin, vancomycin, chlorhexidine and gentamicin in multidrug-resistant strains. To enhance the antibacterial activity and the half-life of melectin (MEP, GFLSILKKVLPKVMAHMK-NH_2), which exhibited high antimicrobial activity against Gram-positive and Gram-negative bacteria at MIC values from 4 to 120-times lower than the HC_{50} value, Niederhafner et al. (2010) reported its dendrimerization [2]. In the study, 23 dendrimer derivatives of melectin have been synthetized and evaluated for their antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* species as well as for their hemolytic toxicity. According to the results, in contrast with the findings reported by Young et al., except for some cases, in which HC_{50} increased and therefore the hemolytic activity reduced, it was generally superior [2]. On the other hand, their antibacterial activity against *B. subtilis*, *E. coli* and *P. aeruginosa* was generally improved and anyway some very appealing materials proved very favorable HC_{50}/MICs ratios [2]. Curiously, the antibacterial activity against *S. aureus*, except for three cases, decreased among the dendrimer derivatives. Collectively, the ranges of both MICs and HC_{50} values are very wide, depending on the levels of multivalence and some modification in the peptide sequence made up by the authors, affecting the cationic character of the prepared peptide Ds.

Later, the in vitro activity of the third-generation antimicrobial peptide dendrimer containing the dipeptide sequences KL, named G3KL, was evaluated against 32 *A. baumannii* strains, including 10 OXA-23, 7 OXA-24, and 11 OXA-58 carbapenemase producers isolates and against 35 *P. aeruginosa* strains, including 18 VIM and 3 IMP carbapenemase producers isolates and the results were compared to the activities of standard antibiotics[3]. The peptide dendrimer showed $MICs_{50/90}$ values of 8/8 μg/mL and $MBCs_{50/90}$ values of 8/8 μg/mL against both species collections, minimal hemolytic concentration of 840 μg/mL vs. 2000 μg/mL for polymyxin B, and stability in human serum, being its half-life [$t_{1/2}$] of 18 h.

A series of eight amphiphilic peptide Ds, built up around a dendronized ornithine (Orn) *core*, were synthesized by Polcyn et al. (2013) and evaluated for their antimicrobial properties against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 strains[4]. The achieved peptide Ds showed very different MIC values: two Ds proved low activity against all the bacteria, four Ds proved appreciable activity only against *S. aureus* ATCC 25923 strain, and the residual two manifested good activity against all the bacteria, with mild effects against *P. aeruginosa*. A higher antimicrobial potency was correlated with a higher charge density and branching and to a higher lipophilicity of the residues located at the C-terminus. In particular, the most efficient peptide Ds were the isomeric hexachlorides, namely 3d and 3h, whose structure presented a C12 lipophilic chain, due to the dodecylamine residue. Of the two structures, dendrimer 3d, which possesses cationic amine groups less cluttered and free to interact electrostatically with the bacterial membrane, has shown MIC values of 4, 0.99, 4, and 16.9 μg/mL respectively, against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300, *E. coli* ATCC 25922 AND *P. aeruginosa* ATCC 27853 strains. Experiments on hemolytic toxicity on RBCs showed a strict correlation with the antimicrobial activity of the Ds and their structural properties. In fact, the compounds that were practically inactive against bacteria, were also the less toxic. Unexpectedly, compound 3d, presenting the best performance in terms of antibacterial activity, caused only about 35% of hemolysis when used at 100 μM concentration.

A series of peptide Ds were synthetized by Stach et al. (2012) and investigated for their antimicrobial activity against *B. subtilis*, *E. coli* and *P. aeruginosa* species and for their hemolytic toxicity[5]. Although some compounds were totally inactive and a limited activity was generically observed against *P. aeruginosa*, the compound (H1), with sequence Leu8(Lys-Leu)4(Lys-Phe)2Lys-LysNH_2 (Lys = branching lysine) and the analog compound (bH1), with sequence Leu8(Dap-Leu)4(Dap-Phe)2Dap-LysNH2 (Dap = branching 2,3-diaminopropanoic acid) showed good antimicrobial activities, mediated by membrane disruption. In particular, bH1 was the most potent as an antimicrobial, and more active than Indolicidin (ILPWKWPWWPWRRNH_2) against all the bacteria tested. Moreover, it showed a good antibacterial activity also against *P. aeruginosa* (MIC values = 20 μg/mL). In addition, bH1 displayed very low hemolytic activity, with a minimal hemolytic cytotoxicity (MHC) ≥ 2000 μg/mL. H1 and bH1 were also tested against strains of *S. aureus*, *S. epidermidis* ATCC 14990, *En. faecium* and *E. coli* species. The results showed MIC values in the range 8→32 μg/mL and in some cases their activity was higher than that of Polymixin B and/or Ampicillin.

Scorciapino et al. (2012) prepared a semisynthetic dendrimer (dimeric) peptide (SB056), by alternating hydrophilic and hydrophobic amino acids, achieving a membrane-active peptide able to form amphiphilic β-strands in a lipid environment[6]. Lipid monolayer surface pressure experiments revealed that SB056 exerted its membranolytic activity by a

sort of lipid-induced aggregation mechanism. SB056 showed high activity against multidrug-resistant Gram-negative bacteria comparable to that of colistin and polymyxin B, with an even broader spectrum of activity than numerous other reference compounds. On the contrary, its activity on Gram-positive bacteria was more limited.

In a subsequent study, investigations into the antibiofilm activity and hemolytic toxicity of SB056 were carried out. According to the results, the peptide dendrimer provided a HC_{50} value of 159 μg/mL, thus asserting a low level of hemolytic toxicity and its potentials as a therapeutic alternative to conventional antibiotics to treat infections by multidrug-resistant bacteria. With regard to its antibiofilm activity, the antimicrobial activity of SB056 was first investigated against planktonic forms of reference strains of *S. epidermidis* and *P. aeruginosa* under biofilm-like conditions, unfortunately obtaining very high MIC values (≥102.4 μg/mL). Then, the ability of the peptide to inhibit biofilm formation was evaluated against the same strains. When tested at 25.6 μg/mL on *S. epidermidis*, SB056 exhibited strong antibiofilm activity, causing a reduction of approximately 98% of the biofilm biomass. When it was tested against *P. aeruginosa*, SB056 caused a 90% reduction of biofilm biomass and only at the concentration of 51.2 μg/mL.

Later, the same group amplified their studies concerning the SB056 dendrimer peptide (in this new study named den-SB056), enhancing the amphiphilic profile of the original sequence [WKKIRVRLSA-NH_2] by interchanging the first two residues [KWKIRVRLSA-NH_2] and obtaining den-SB056-1[7]. Results obtained against *E. coli* and *S. aureus* planktonic strains confirmed a reduced activity on *E. coli* (MICs = 16 μg/mL vs. 8 μg/mL of the native dendrimer) and an unchanged activity on *S. aureus* (MICs = 32 μg/mL) associated with increased hemolytic toxicity (HC_{50} = 87 μg/mL). Concerning bactericidal activity against the same strains, the modified dendrimer peptide was less active on *E. coli* (MBC = 16 vs. 8 μg/mL), whereas it was more active on *S. aureus* (MBC = 32 vs. 64 μg/mL). Nonetheless, the efficacy of den-SB056-1 was higher than that of den-SB056 both against Gram-positive and Gram-negative bacteria, specifically when tested in broths supplemented with physiological concentration of electrolytes. Results obtained from the evaluation of den-SB056-1 activity on sessile forms of *S. epidermidis* and *P. aeruginosa* bacteria established a remarkable reduced activity in decreasing biofilm biomass, especially if compared to the same activity possessed by the native den-SB056[6].

Recently, the group of Scorciapino, reconsidered the small peptide dendrimer (den-SB056) and its more hydrophobic modified derivative (den-SB056-1). Aiming to increase their multivalence, two copies of den-SB056 or of den-SB056-1 were bound to the α- and ε-nitrogen atoms of one lysine *core*. Finally, an 8-aminooctanamide residue was added at the C-terminus of the lysine to improve its cationic character and its membrane affinity[8].

A second-generation peptide dendrimer (2D-24), containing residues of lysine and tryptophan, was synthetized by Bahar et al. (2015) by solid phase method and its bioactivity was investigated against planktonic, biofilm, and persister cells of the wild-type *P. aeruginosa* (PAO1) and its mucoid mutant strain (PDO300)[9]. 2D-24 was found to definitely kill planktonic cells of both strain types at concentrations of 77.5 μg/mL and to kill 94% of their biofilm cells at concentrations of 46.5 μg/mL, suggesting that 2D-24 is able to penetrate the biofilm matrix and the alginate layer of the mucoid strain. Concentrations up to 310 μg/mL of 2D-24 were necessary to kill 69 and 89% of multidrug tolerant persister cells of PAO1 and PDO300, respectively. Although such a high concentration establishes a total inactivity of the dendrimer against persister cells, the synthetic peptide proved a promising synergistic effect when administered in combinations with ciprofloxacin, tobramycin, or carbenicillin. Based on hemolysis assays, using sheep erythrocytes and on cytotoxicity tests, using a coculture model of PAO1 and IB3-1 human epithelial cells, 2D-24 exhibited very low levels of hemolytic toxicity (HC_{50} > 1000 μg/mL) and was found to kill *P. aeruginosa* cells at concentrations that are not toxic to mammalian cells (25 μg/mL).

Stach and colleagues (2014), inspired by the prevalence of leucine and lysine residues in several natural CAMPs, prepared six third-generation *L*-peptide Ds, namely G3KL, G3LK, G3KK1, G3KK2, G3LL1 and G3LL2 by solid phase peptide synthesis (SPPS)[10]. The investigation results on their potency against *P. aeruginosa*, *E. coli*, and *B. subtilis* established that G3KL, with the sequence (KL)8(KKL)4(KKL)2KKL, showed a good broad spectrum of activity, with high selectivity for bacteria cells and low hemolytic properties. Further investigations on dendrimer G3KL and on its enantiomer (DG3KL) were performed in the presence of human serum, which represents a degradation factor for natural CAMPs, thus limiting their activity in vivo. Both Ds proved high antimicrobial activity against *P. aeruginosa* PAO1, reporting MIC values of 2 μg/mL, with partial degradation (60% after 24 h) for DG3KL, and of 0.5 μg/mL, with no degradation after 24 h for DG3KL. G3KL and DG3KL were also tested against four clinical isolates of *P. aeruginosa*, resistant to at least two different classes of antibiotics, including β-lactams, aminoglycosides or quinolones, and also against several reference strains, such as *A. baumannii* ATCC19606, *E. coli* W3110, and *E. aerogenes* 13048 and against lipopolysaccharide (LPS) mutant strains of *P. aeruginosa*. According to the results, the peptide Ds showed MIC values in the range 2–32 μg/mL on all resistant strains of *P. aeruginosa* and reference strains of *A. baumannii* and *E. coli* examined. They showed lower activity on *E. aerogenes* (MIC values = 64 and 32 μg/mL, for the two Ds respectively), but comparable activity against LPS mutant strains of *P. aeruginosa*.

Recently, the *L*-peptide dendrimer G3KL was additionally taken into consideration by the group of Siriwardena[11]. By using virtual screening techniques in the field of Ds and a chemical-space-guided approach for the first time to search for new and improved analogs of G3KL, from a very large virtual library of compounds, Siriwardena et al. (2018) identified the dendrimer peptide T7 as the most improved one. T7 showed significant action across all the strains tested, through a membrane disruption mechanism, combined with excellent stability in the presence of serum, and negligible hemolytic activity. T7 proved expanded activity range against Gram-negative pathogenic bacteria including *K. pneumoniae* and promising activity in an in vivo infection model by a multidrug-resistant strain of *A. baumannii*. Due to their studies, the authors established that dendrimer size does not limit the activity of dendrimer peptides.

The following year, the Siriwardena group[12] obtained a new peptide dendrimer (DC5) including in its structure both the outer branches of a peptide dendrimer active against *P. aeruginosa*, *A. baumannii* and methicillin-resistant *S. aureus* (TNS18), and the *core* of T7, previously reported, and active against *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. DC5 was able to display the activity features of both parent compounds and retained a similar mechanism of action. In particular, DC5 exerted antimicrobial activity against PAO1 at very low MIC values, both in the presence or in the absence of human serum (MIC values = 2–4 μg/mL) and presented a good activity against multi drugs resistant (MDR) strains of Gram-negative and Gram-positive bacteria (MIC values in the range of 4–32 μg/mL) and low levels of hemolytic toxicity (MHC = 500 μg/mL).

Together with three linear peptides [(RW)n-NH_2, where n is 2, 3, or 4], a dendrimer peptide, $(RW)_{4D}$ was synthetized by Chen et al. (2011)[13]. The bactericidal activity of $(RW)_{4D}$ was tested against planktonic persister cells, planktonic regular cells, and persister cells in preformed biofilms of the *E. coli* HM22 strain. Against planktonic persister cells, a dose-dependent killing activity was observed and significant killing was detected already at a concentration of 20 and 40 μM of the dendrimer peptide, which showed a reduction in the number of viable cells by a half log and one log, respectively. Unexpectedly, the exposure of *E. coli* cells to a concentration of 80 μM of $(RW)_{4D}$ only led to a decrease of 2-log in the killing activity. When normal *E. coli* cells were exposed to $(RW)_{4D}$, a similar scenario was observed. Interestingly, when the bactericidal activity of $(RW)_{4D}$ was evaluated on *E. coli* HM22 persister cells residing in 24 h sessile biofilms preformed on 316L stainless steel coupons, a potent dose-dependent dispersion of biofilm cells was detected. In particular, after treatment for 1 h with concentrations of $(RW)_{4D}$ equal to 20, 40 and 80 μM, the total number of viable biofilm cells were reduced by 77.1%, 98.8% and 99.3% respectively, compared to the untreated control. In addition, the same treatments reduced, the tolerance of the biofilm cells to ampicillin by 57.2%, 96.9% and 99.1% respectively. Furthermore, no viable cells were found after the biofilms were treated with concentrations of $(RW)_{4D}$ of 40 or 80 μM, followed by 5 g/mL ofloxacin, suggesting that all persister cells were eliminated.

Interestingly, tissue attachment and biofilm formation due to *P. aeruginosa*, is mediated, among other factors, by two lectins, LecA and LecB, that bind specifically to galactosides and fucosides. Therefore, a successful approach to treat infections caused by *P. aeruginosa* and to inhibit biofilm formation and/or to disperse established biofilm, may include the treatments with lectin-binding saccharide solutions[14]. In this regard, the Kadam group reported the synthesis and the evaluation of the effectiveness of lectin inhibitors in the form of glycopeptide Ds, with four fucosides (FD2) or four aromatic galactosides and aliphatic thiogalactosides (GalAG2 and GalBG2)[15][16] at the end of a common peptide dendrimer scaffold. Except for GalBG2, they were all able to bind tightly to their respective lectins and, consequently, to block formation and to induce dispersion of *P. aeruginosa* biofilms. Subsequently, Kadam and colleagues investigated several amino acid sequence variations for FD2[17][18] and for GalAG2[14], which were found to modulate the lectin-binding affinity, the solubility of the Ds, and the capability to inhibit biofilm formation.

More recently, Visini et al. (2015) reported structural studies concerning galactoside Ds of type GalA, capable of binding to *P. aeruginosa* LecA and showed that lectin aggregation is necessary for biofilm inhibition. At the same time, these studies have established the importance of the multivalence of glycopeptide Ds as a unique opportunity to control *P. aeruginosa* biofilms[19].

Based on the results obtained by Visini et al. in their studies, the year after, Bergman et al. (2016), prepared various G3 and G4 analogs of GalAG2 and GalBG2 using the multiple chloroacetyl cysteine (ClAc) thioether ligation as the key step. By using different approaches such as essays of inhibition of hemagglutination, isothermal titration calorimetry tests and biofilm inhibition evidence, the authors showed that G3 Ds bound LecA slightly better than their parent G2 Ds and induced complete inhibition and dispersal of biofilms of *P. aeruginosa*, while G4 Ds showed reduced binding and no biofilm inhibition capabilities[20].

That same year, Michaud, Visini, Bergaman et al., extended previous research on glycopeptide Ds, through further synthetic variations and activity combinations approaches, to obtain more potent biofilm inhibitors[21]. First, the multivalent chloroacetyl cysteine thioether ligation was used as an efficient method to build heteroglycoclusters including Het1G2-

Het8G2 and $(Het2G1\text{-}Cys)_2$ targeting both LecA and LecB. Subsequently, in a second approach, LecB targeting glycopeptide Ds, displaying analogs of the Lewis^a antigen (Le^{a}CxG2) and β-fucoside derivative (FucC6G2), were investigated. In addition, to test the antimicrobial effects of the cationic peptide backbones contained in Het1G2-Het8G2 alone, non-glycosilated peptide Ds were prepared as controls. Heteroglycoclusters incorporating cationic residues displayed enhanced biofilm inhibition capability, associated with bactericidal behavior similar to that of membrane disrupting polycationic Ds. In particular, the best performing Het7G2 dendrimer showed an MBIC value of 13 μM, an MBC value of 13 μM and the ability to disperse 100% of the already established biofilms, at a concentration of 50 μM. Analogous Ds of the Lewis^a antigen, a natural LecB ligand, though stronger LecB ligands, displayed a decrease in biofilm inhibition, when compared with parent dendrimer FD2 (MBIC = 30 μM and approximately 88% dispersal at 50 μM). On the contrary, FuC6G2 showed a slightly better capacity to inhibit both biofilm formation (MBIC = 9 μM vs. 20 μM) and its dispersion (100% dispersal at 30 μM vs. 50 μM). However, from evaluations of non-glycosilated peptide Ds, it was observed that two Ds of this series (AcG2xK and NG2) were, one comparable with Het7G2 (AcG2xK) and the other the most active compound in terms of biofilm formation inhibition activity (MBIC = 13 and 2.5 μM) in killing bacteria under biofilm formation conditions (MBC = 13 and 2.5 μM) and in dispersing the established biofilm (100% dispersal at 50 μM for AcG2xK and at 22 μM). Finally, the authors proved that the synergistic application of the LecB specific non-bactericidal antibiofilm dendrimer FD2, and the antibiotic tobramycin (both compounds applied at sub-inhibitory concentrations), allowed effective biofilm inhibition and dispersal. In particular, strong synergistic effects were observed from the co-administration of FD2 with 0.1 μM Tobramycin, with new MBIC values of 5 μM, associated with a 100% dispersal of the established biofilm at a concentration of 10 μM.

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