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In Vitro Activity of the Novel Antimicrobial Peptide Dendrimer G3KL against Multidrug-Resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

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The *in vitro* activity of the novel antimicrobial peptide dendrimer G3KL was evaluated against 32 *Acinetobacter baumannii* (including 10 OXA-23, 7 OXA-24, and 11 OXA-58 carbapenemase producers) and 35 *Pseudomonas aeruginosa* (including 18 VIM and 3 IMP carbapenemase producers) strains and compared to the activities of standard antibiotics. Overall, both species collections showed $MIC_{50/90}$ values of 8/8 μg/ml and minimum bactericidal concentrations at which 50% or 90% of strains tested are killed ($MBC_{50/90}$) of 8/8 μg/ml. G3KL is a promising molecule with antibacterial activity against multidrug-resistant and extensively drug-resistant *A. baumannii* and *P. aeruginosa* isolates.

The spread of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates that are resistant to carbapenem antibiotics due to the production of carbapenemases represents a serious threat (1). These strains are usually multidrug-resistant (MDR) due to the coexpression of mechanisms involving other classes of antibiotics, thus drastically limiting our therapeutic armamentarium (2–4). In particular, extensively drug-resistant (XDR) isolates are commonly detected worldwide (5), whereas the prevalence of pandrug-resistant (PDR) isolates is increasing worryingly in several countries (6–8). Therefore, novel antimicrobial strategies need to be rapidly developed.

Recently, there has been a rising interest in evaluating naturally occurring or synthetic antimicrobial peptides (AMPs) with activity against prokaryotic membranes. This attention is due to their wide spectrum of activity against both Gram-positive and Gram-negative species, potent bactericidal activity, and ability to bypass common mechanisms of resistance that affect standard antibiotics (9, 10). However, several reasons have so far limited the clinical implementation of AMPs: (i) high susceptibility to degradation by endogenous and microbial proteases; (ii) toxicity due to the high concentration necessary to inhibit bacteria; and (iii) short half-life because of high protein binding (11). Several authors have modified AMPs to obtain proteolytically resistant versions, mostly by sequence variations and the use of D-amino acids (12–15). However, redesigning the peptide chain topology, in particular by introducing multiple branching points to obtain synthetic AMP dendrimers (AMPDs), seems a promising solution to overcome all of the the aforementioned problems (16–18).

G3KL is a novel AMP dendrimer (AMPD) developed at the Department of Chemistry and Biochemistry of the University of Bern (Switzerland) by sequence optimization of an initial hit compound identified by screening a combinatorial library of dendrimers using a tailored high-throughput screening assay and presumed to act as a membrane-disrupting agent (19–22). Its activity requires a dendritic topology and only natural lysine and leucine residues alternating in the branches (Fig. 1). This novel AMPD has demonstrated *in vitro* activity against several Gram-negative strains, low toxicity to human red blood cells (minimal hemolytic concentration of 840 μg/ml versus >2,000 μg/ml for polymyxin B), stability in human serum (half-life [$t_{1/2}$] of ~18 h and MICs of 2 and 4 μg/ml for *P. aeruginosa* PAO1 in Mueller-Hinton medium with or without 30% human serum, respectively), and easy preparation by standard solid-phase peptide synthesis; attempts to identify G3KL-resistant strains were also unsuccessful (21).

In the present work, we analyzed the *in vitro* activity of G3KL against 32 *A. baumannii* and 35 *P. aeruginosa* isolates collected in different countries during diverse periods. Species identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker), whereas MICs for different classes of antibiotics were obtained in cation-adjusted Mueller-Hinton II (CAMHII) broth (BBL) using the microdilution GNX2F panels (Trek Diagnostics Systems). MICs were interpreted according to the CLSI criteria (23). *P. aeruginosa* ATCC 27853 was used as the control. For G3KL, MICs were also achieved in microdilution in CAMHII broth using both polystyrene and polypropylene 96-well plates (24). The minimum bactericidal concentration (MBC) for G3KL was then obtained by culturing 30 μl of the broth from the endpoint well and from the $\log_2$ dilution above the MIC onto CAMHII agar plates (BBL) (25). For several strains, carbapenemase genes were already characterized (26–29); the presence of β-lactamase genes (including class A,

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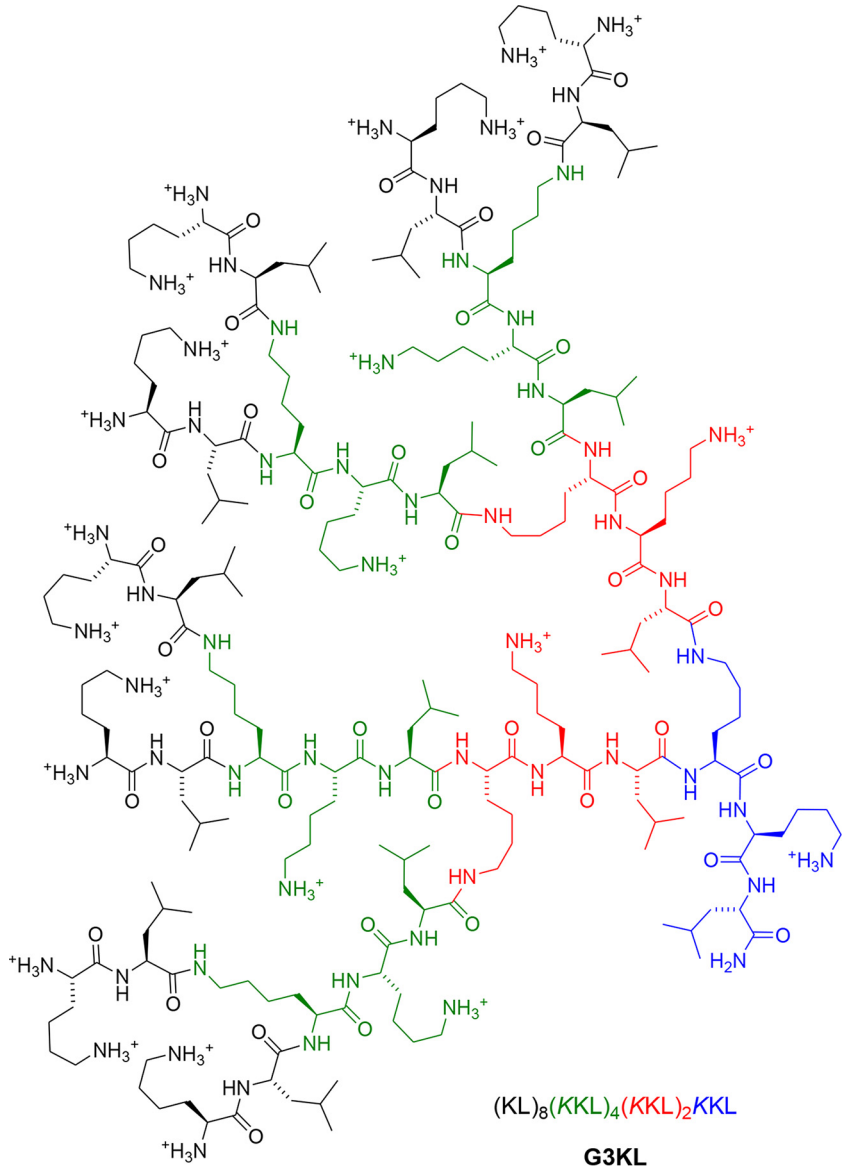

FIG 1 Molecular structure of the novel antimicrobial peptide dendrimer (AMPD) G3KL [amino acid sequence $(KL)_8(KKL)_4(KKL)_2KKL$, where K is lysine, *K* is branched lysine, and L is leucine].

B, and D carbapenemases) in the remaining isolates was analyzed by implementing the CT103XL microarray (CheckPoints). Phenotypic analysis indicated that among the 32 *A. baumannii* isolates, there were 5 MDR, 24 XDR, and 1 PDR isolate, whereas the 35 *P. aeruginosa* isolates included 8 MDR, 19 XDR, and 2 PDR strains (5). Moreover, several of the the strains tested produced the most frequently detected class D (10 OXA-23, 7 OXA-24, and 11 OXA-58) and class B (18 VIM and 3 IMP) carbapenemases described in *A. baumannii* and *P. aeruginosa*, respectively (see Tables S1 and S2 in the supplemental material) (1). As shown in Table 1, the isolates of both species possessed very high MICs for β-lactams (e.g., MIC_{50} values for meropenem of ≥16 μg/ml), aminoglycosides (e.g., MIC_{50} values for gentamicin of ≥16 μg/ml), and ciprofloxacin (MIC_{50} values of ≥4 μg/ml); the only standard antibiotics maintaining *in vitro* activity (≥80%) were colistin and polymyxin B.

For G3KL, polypropylene plates were used to avoid any hypothetical attachment (and therefore effect on the MICs) of the positively charged AMPD to the surface of the polystyrene wells (21). Overall, G3KL showed $MIC_{50/90}$ values of 8/8 μg/ml and MBCs at which 50% or 90% of strains tested are killed ($MBC_{50/90}$) of 8/8 μg/ml for both *A. baumannii* and *P. aeruginosa* strains regardless of the plate used, though slightly lower MIC/MBC ranges were observed for polypropylene plates (Table 1). Given the fact that the recorded MBCs were mostly equal to the corresponding MICs (overall, 64 out of 67 strains tested with polypropylene plates and 51 out of 67 strains tested with polystyrene plates; see Tables S1 and S2 in the supplemental material), G3KL has a putative bactericidal effect (30).

Previous studies have assessed the MICs and MBCs of modified AMPs against *A. baumannii* and *P. aeruginosa*, these being in general higher than those obtained for G3KL (e.g., for *P. aerugi-*

TABLE 1 Overall phenotypic results for standard antibiotics and G3KL antimicrobial peptide dendrimer tested against the collection of 67 isolates

	MIC (or MBC where indicated) (μg/ml) and susceptibility results for isolates of:							
	A. baumannii ($n = 32$)[a]				*P. aeruginosa* ($n = 35$)[b]			
Antimicrobial	MIC_{50}	MIC_{90}	MIC range	S (%)[c]	MIC_{50}	MIC_{90}	MIC range	S (%)[c]
Piperacillin-tazobactam	≥128	≥128	≤8–≥128	9.4	≥128	≥128	≤8–≥128	25.7
Ticarcillin-clavulanate	≥256	≥256	≤16–≥256	6.3	≥256	≥256	≤16–≥256	8.6
Ceftazidime	≥32	≥32	1–≥32	6.3	≥32	≥32	≤1–≥32	20.0
Cefepime	16	≥32	≤2–≥32	25.0	≥32	≥32	≤2–≥32	25.7
Aztreonam	≥32	≥32	16–≥32	NA	≥32	≥32	≤2–≥32	17.1
Imipenem	≥16	≥16	≤1–≥16	15.3	≥16	≥16	≤1–≥16	25.7
Meropenem	≥16	≥16	≤1–≥16	11.5	≥16	≥16	≤1–≥16	25.7
Doripenem	≥4	≥4	0.25–≥4	18.8	≥4	≥4	≤0.125–≥4	25.7
Gentamicin	≥16	≥16	≤1–≥16	6.3	≥16	≥16	≤1–≥16	42.9
Amikacin	≥64	≥64	≤4–≥64	18.8	16	≥64	≤4–≥64	51.4
Ciprofloxacin	≥4	≥4	≤0.25–≥4	6.3	≥4	≥4	≤0.25–≥4	31.4
Tigecycline	1	4	≤0.25–4	NA	8	≥16	2–≥16	NA
Colistin	0.5	1	≤0.25–≥8	96.9	2	≥8	0.5–≥8	80.0
Polymyxin B	0.5	2	≤0.25–≥8	90.6	1	≥8	1–≥8	82.9
G3KL								
MICs	8 (8)[d]	8 (8)	4–16 (4–8)	NA	8 (8)	8 (8)	4–64 (2–32)	NA
MBCs	8 (8)	8 (8)	4–16 (4–8)	NA	8 (8)	8 (8)	4–128 (4–32)	NA

[a] Includes 5 MDR isolates (4 OXA-58 producers and 1 carbapenemase negative), 24 XDR isolates (10 OXA-23, 6 OXA-24, and 7 OXA-58 producers and 1 carbapenemase negative), and 1 PDR isolate (OXA-24 producer) (see Table S1 in the supplemental material).
[b] Includes 8 MDR isolates (2 VIM and 1 IMP producer and 5 carbapenemase negative), 19 XDR isolates (15 VIM and 2 IMP producers and 2 carbapenemase negative), and 2 PDR isolates (1 VIM producer and 1 carbapenemase negative) (see Table S2 in the supplemental material).
[c] S, susceptible according to CLSI criteria of 2014 (23); NA, not applicable.
[d] Results in parentheses are those obtained with polypropylene 96-well plates.

nosa, MICs of 150 and MBCs of 20 to >300 μg/ml were reported in references 12 and 13). However, in one study, five polycationic AMPs were tested against *A. baumannii*, and MIC_{50} values similar to those found here for G3KL were obtained, although the MIC_{90}/MBC_{90} values (both ranged from 16 to 32 μg/ml) were overall higher than for G3KL (31). In another study, 15 AMPs were tested against colistin-resistant and -susceptible *A. baumannii* strains, and only a few yielded $MIC_{50/90}$ values similar to those for G3KL; moreover, many had significantly different MICs for the two groups of isolates, a phenomenon that we observed for G3KL (Table 1) (14). Sanchez-Gomez et al. assessed 11 AMPs against *P. aeruginosa*, with only one lactoferrin analogue having $MIC_{50/90}$ and $MBC_{50/90}$ values equal to those of G3KL (15). However, since this is a derivative of a naturally occurring linear peptide, *in vivo* proteolysis is likely to happen. Very recently, several dendrimers have also been evaluated. Lind et al. reported MICs of 41 to 149 μM (i.e., ~88 to 300 μg/ml) for three dendrimers against *P. aeruginosa* ATCC 27853 (32), whereas Bahar et al. observed no viable cells of *P. aeruginosa* PAO1 and PDO300 strains after treating them with a dendrimer at a concentration of 50 μM (i.e., ~77 μg/ml) (33).

Overall, we note that there is a scarcity of data regarding the *in vitro* performance of recently designed AMPDs against *P. aeruginosa* and *A. baumannii*. More importantly, for most of the aforementioned studies (with either AMPs or AMPDs), small numbers of strains or only laboratory controls were tested. In contrast, we evaluated the activity of G3KL against a large collection of difficult-to-treat isolates (including those producing carbapenemases) that are frequently faced in the contemporary international clinical scenario.

In conclusion, the AMPD G3KL possesses promising *in vitro* activity against *A. baumannii* and *P. aeruginosa*, yielding results that are not apparently different for wild-type, MDR, XDR, and PDR isolates (either producing or not producing carbapenemases). Since G3KL does not resemble other molecules in nature, has low MIC/MBC values compared to those of other AMPs and AMPDs, and has little toxicity for red blood cells *in vitro*, it seems plausible that this molecule should be further investigated with tissue culture assays and animal studies to support future pharmacological formulations and potential clinical applications.

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