Rational modification of a dendrimeric peptide with antimicrobial activity: consequences on membrane-binding and biological properties

Giovanna Batoni,[a] Mariano Casu,[b] Andrea Giuliani,[c] Vincenzo Luca,[d] Giuseppantonio Maisetta,[a] Maria Luisa Mangoni,[d] Giorgia Manzo,[e] Manuela Pintus,[e] Giovanna Pirri,[c] Andrea C. Rinaldi[e,#], Mariano A. Scorciapino,[e] Ilaria Serra,[e] Anne S. Ulrich,[fg] Parvesh Wadhwani,[f]

Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy[a]; Department of Chemical and Geological Sciences, University of Cagliari, Cittadella Universitaria, Monserrato (CA), Italy[b]; Research & Development Unit, Spider Biotech S.r.l., Colleretto Giacosa (TO), Italy[c]; Dipartimento di Scienze Biochimiche, "A. Rossi Fanelli", Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza Università di Roma, Rome, Italy[d]; Department of Biomedical Sciences, University of Cagliari, Cittadella Universitaria, Monserrato (CA), Italy[e]; Institute of Biological Interfaces (IBG-2), Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany[f]; Institute of Organic Chemistry, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany[g]

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#Address correspondence to Andrea C. Rinaldi, rinaldi@unica.it

Abstract

Peptide-based antibiotics might help containing the rising tide of antimicrobial resistance. We developed SB056, a semi-synthetic peptide with a dimeric dendrimer scaffold, active against both Gram-negative and -positive bacteria. Being the mechanism of SB056 attributed to disruption of bacterial membranes, we enhanced the amphiphilic profile of the original, empirically derived sequence [WKKIRVRLSA-NH_2] by interchanging the first two residues [KWKIRVRLSA-NH_2], and explored the effects of this modification on the interaction of peptide, both in linear and dimeric form, with model membranes and on antimicrobial activity. Results obtained against *E. coli* and *S. aureus* planktonic strains, with or without salts at physiological concentrations, confirmed the added value of dendrimeric structure over the linear one, especially at physiological ionic strength, and the impact of the higher amphipathicity obtained through sequence modification on enhancing peptide performances. SB056 peptides also displayed intriguing anti-biofilm properties. *S. epidermidis* was the most susceptible strain in sessile form, notably to optimized linear analog lin-SB056-1 and the wild-type dendrimer den-SB056. Membrane affinity of all peptides increased with the percentage of negatively charged lipids, and was less influenced by the presence of salt in the case of dendrimeric peptides. The analog lin-SB056-1 displayed the highest overall affinity, even for zwitterionic PC bilayers. Thus, in addition to electrostatics, distribution of charged/polar and hydrophobic residues along the sequence might have a significant role in driving peptide-lipids interaction. Supporting this view, dendrimeric analog den-SB056-1 retained greater membrane affinity in the presence of salt than den-SB056, despite they bear exactly the same net positive charge.

INTRODUCTION

Antimicrobial resistance is a growing public health threat at the global level. A wide range of infectious agents have developed resistance against most classes of clinically usable antibiotics, making the choices of alternative treatments very limited or simply non-existent (1, 2). Alarming rates of multi-drug resistance in bacterial strains that cause common healthcare-associated and community-acquired infections have been reported worldwide. Furthermore, many important fungal, protozoan and viral pathogens are also increasingly resistant to currently available therapies. For example, artemisinin resistance in malaria is spreading, and increasing levels of transmitted anti-HIV drug resistance have been detected among patients starting antiretroviral treatment (3). "A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century," commented a recent WHO report on antimicrobial resistance (3). Besides the health burden that this situation poses, the wider societal and economic impact of antimicrobial resistance is also of alarming proportions.

Although antimicrobial resistance is a complex phenomenon due to a host of circumstances – not least the natural tendency of microorganisms to develop it – it is certainly accelerated by the selective pressure exerted by use and misuse of antimicrobial agents in both humans and animals. In addition to the need to protect the efficacy of existing drugs through a wiser use of their properties, the paucity of new antimicrobial agents on the horizon to replace those that have become ineffective, calls for the urgent quest for new antibiotic substances to be developed and deployed in the clinic.

Antimicrobial peptides (AMP) – also known as host defense peptides – have shown promise to be these new class of antibiotics, with some of them having already successfully made their way to the clinic such as gramicidin S and polymixin B, and many more peptidic molecules that are in developmental stage to replenish the waning arsenal of antibiotics. This

is a widely distributed group of molecules expressed by multicellular organisms as effectors of the innate immune system, making the first line of host defense against invading pathogens. AMPs are characterized by a wide spectrum of antimicrobial activity, ranging from Gram-positive to Gram-negative bacteria (including, in several instances, bacteria resistant to conventional antibiotics), from fungi to enveloped viruses and protozoa (4-6). Largely diverse in sequence and secondary structures, AMPs generally share a cationic character and an amphipathic fold. Whereas conventional antibiotics interact with specific bacterial targets, e.g. inhibiting key enzymes, thus allowing the pathogens to develop resistance relatively easily, AMPs usually act by physically destroying or permeabilizing the microbial plasma membrane through unspecific interactions with the lipids (7, 8). In this case, target substitution/modification, and thus resistance, is less likely to occur, making AMPs and their derivatives particularly suitable as novel antimicrobial drugs, at least in theory. That is, if an AMP is designed to exclusively act by destroying the bacterial membrane without permeating inside the bacterial cell to other possible targets, then the development of the resistance is less likely to occur. Given the multi-functionality of membrane active peptides, their intrinsic metabolic unstability and the associated delivery issues, only a handful of peptides have been clinically approved so far, and mostly for external, topical use. Multiple hurdles limit the direct development of naturally occurring AMPs into clinically useful antibiotics, indeed. These include susceptibility to protease degradation and a reduced activity in the presence of salts at physiological concentrations; and high manufacturing costs are also of considerable concern (9, 10).

Since the direct use of naturally occurring AMPs has resulted in poor therapeutic application, alternative approaches have been pursued to overcome the inherent limitations of these molecules. The modification of existing peptide sequences to make them proteolytically more stable, and the *de novo* synthesis of peptides and/or the design of synthetic molecules

mimicking the properties and activities of natural AMPs, have both being attempted (11, 12). To reduce the economical cost of production and to avoid the associated immune response, significant efforts have been focused on developing shorter, active, less hemolytic sequences and modifications thereof. In particular, dendrimeric peptides have received much attention in recent years, as they proved promising candidates for a number of applications. Peptide-based dendrimers are branched macromolecules consisting of a core and a certain number of covalently attached functional units (13). Dendrimeric peptides usually display increased activity compared to their monomeric counterparts, probably because of the higher local concentration of bioactive units. In addition, short peptides synthesized in oligodendrimeric form often show high resistance to proteolytic degradation, probably because of protection against protease action offered by steric hindrance, thus increasing the peptides' pharmacokinetic properties and making them suitable for use *in vivo* (14). A range of peptides with different sequence and oligodendrimeric design was shown to display broad-spectrum activity against microbial pathogens – including Gram-positive/negative bacterial strains and selected viruses – and also the ability of targeting and killing cancer cells (15-20).

Rational modifications of the sequence of a linear AMP originally identified by selecting a random phage library against *Escherichia coli* cells led to SB056, a novel AMP with a primary sequence [WKKIRVRLSA] capable of forming a dimeric dendrimer scaffold. SB056 is highly active against Gram-negative bacteria, with potency comparable to that of the clinically used colistin and polymyxin B, but it shows a broader spectrum of activity, with an interesting activity also against Gram-positive bacteria (21). A thorough biophysical characterization, combined with membrane affinity assays, showed that this interesting peptide was indeed membrane-active by folding into an amphiphilic β-type conformation in lipidic environments (21). Having observed the β-type conformation of SB056, further modification of the peptidic part of the dendrimeric SB056 by interchanging the first two

residues was then performed to promote a more regular β-type conformation with a full pattern of alternating hydrophilic and hydrophobic amino acids, and thus a perfectly regular amphipathic analogue [KWKIRVRLSA] was designed (22). Intriguingly, such enhancement of the amphipathic profile led to the formation of more ordered and stable β-strands when the monomeric, linear peptide bound to model membranes and to an increased antimicrobial performance against both Gram-positive and -negative bacterial strains (22).

Our preliminary observations on the linear component of SB056 and its amphipathically-enhanced analogue (22) were greatly expanded in the present study. In particular, we report on more recent investigations that have explored the effects of amphiphilic profile enhancement in the context of a dendrimeris (dimeric) scaffold, especially focusing on the resulting antimicrobial activity. Noteworthy, activity of both linear and dendrimeric AMPs was tested against biofilms and in the presence of salts at physiological concentrations, and their interaction with model membranes assayed. Findings confirm the advantages offered by dendrimeric peptides over monomeric, unbranched counterparts, and also support the value of rational modification (enhanced amphipathic profile) of AMPs as driven by detailed information on structure-function relationships.

MATERIALS AND METHODS

Materials. Amino acids and a NovaPEG Rink Amide resin (0.67 mmol/g) were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, USA) and Novabiochem (Merck Chemicals Ltd., Nottingham, UK), respectively. Peptide synthesis grade N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), dichloromethane, diethyl ether and O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU) were purchased from ChemImpex (Wood Dale, IL, USA) and Sigma-Aldrich. PC (1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine) and PG (1-palmitoyl-2-oleyl-*sn*-glycero-3-

phosphoglycerol) sodium salt were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). All other reagents and solvents were purchased from Sigma-Aldrich at the highest available purity and were used with no further purification.

Peptide synthesis. Both the monomeric linear peptides were synthesized with the C-terminus amidated [WKKIRVRLSA-NH_2] and [KWKIRVRLSA-NH_2]. In the following, they will be referred to as lin-SB056 and lin-SB056-1, respectively. Dendrimeric peptides were synthesized as a branched dimer on a lysine scaffold with 8-aminoctanamide (Aoc) as an "anchoring" hydrophobic tail $[WKKIRVRLSA]_2$-K-8Aoc-NH_2 and $[KWKIRVRLSA]_2$-K-8Aoc-NH_2. Thereafter, they will be referred to as den-SB056 and den-SB056-1, respectively. Fig. 1 schematically shows such a dendrimeric structure. A manual standard solid-phase peptide Fmoc (9-fluorenylmethyoxy-carbonyl) strategy was employed, working under nitrogen flow. Coupling reactions with Fmoc-protected amino acids were activated in situ using HBTU, 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIPEA) with a ratio HOBt/DIPEA/HBTU of 1/2/0.9. The branched lysine core was synthesized on the resin by using $(Fmoc)_2$Lys-OH protected amino acid, and the first amino acid on the core was amidated with Aoc. A six fold excess of each Fmoc-protected amino acid was employed in every coupling step of the synthesis, and the following acid-labile protecting groups were used for reactive side chains: 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl for arginine; tert-butyl ether for serine; tert-butyloxycarbonyl for lysine. The Fmoc group was removed by using 20% piperidine in NMP. The other protecting groups were removed during cleavage of the peptide from the solid support by treatment with a TFA/triisopropylsilane/H_2O solution at a 95/2.5/2.5 ratio for 2 h. After cleavage, the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The crude peptides were precipitated from diethyl ether, washed several times with diethyl ether, and dried under reduced pressure. RP-HPLC peptide analysis was performed on a

Jupiter Proteo analytical C12 column (4.6 x 250 mm) supplied by Phenomenex (Torrance, CA, USA), using 0.1% TFA/H_2O as solvent A, and 0.1% TFA/MeCN as solvent B. The column was equilibrated with an A/B ratio of 95/5 at a flow rate of 1.0 mL/min, and the concentration of B was raised to 95% (V/V) over 14 min using gradient mode conditions. The peptide was purified on a Jupiter Proteo semi-preparative C12 column (10 x 250 mm), and the major peak in the chromatogram was collected by an automatic fraction collector. The monoisotopic molecular mass of the dendrimer was determined by MALDITOF MS (Bruker Daltonik, Bremen, Germany), using sinapinic acid as acidic matrix. The instrument was calibrated with peptides of known molecular mass in the 1000-6000 Da range.

Minimal inhibitory concentration and killing kinetic assays. Antimicrobial assays were performed against *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 by using the microbroth dilution method in sterile 96-well plates, according to (23, 24). Briefly, bacteria were grown in Luria-Bertani (LB) medium at 37°C till a mid-log-phase, which was aseptically monitored by absorbance at 590 nm (OD_{590} 0.8). Afterwards, bacterial suspension was diluted in Mueller-Hinton broth (MHB) at a final cell density of $2x10^6$ colony-forming units (CFU)/mL. Aliquots (50 µL) were added to 50 µL of MHB supplemented or not with 150 mM NaCl and containing the peptide at different concentrations. Cells incubated in MHB containing peptide solvent (water) were used as control. Microbial growth was assessed by measuring the absorbance at 590 nm of the plate, after an incubation of 16-18 h at 37°C, using a microplate reader (Infinite M200, Tecan, Salzburg, Austria). The minimal inhibitory concentration (MIC) was defined as the concentration of peptide at which 100% inhibition of microbial growth is observed (25). To determine the bactericidal activity of the peptides and their killing kinetics, 50 µL of the bacterial suspension in MHB ($2x10^6$ CFU/ml) were added to wells of a microtiter plate (each well containing 50 µL of MH broth supplemented with the peptide at different concentration) (26). The plate was incubated at 37°C. At different time

intervals, aliquots of 5 µl were withdrawn from each well, diluted and spread onto LB-agar plates for counting after overnight incubation at 37°C. Control samples were incubated in MHB containing peptide solvent (water). Cell viability was expressed as percentage of survival compared to the control at time zero. The minimal bactericidal concentration (MBC) was defined as the minimal concentration of peptide causing a reduction in the number of viable cells ≥ 3 $\log_{10}$ within 90 min (27).

Biofilm inhibition assay. The antibiofilm activity of dendrimeric and linear peptides were tested against *Staphylococcus epidermidis* ATCC 35984 and *Pseudomonas aeruginosa* ATCC 27853. First, MIC values of peptides against planktonic cells were determined under the same experimental conditions used for the biofilm inhibition assay. To this end, bacterial suspensions were grown overnight in Tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 0.25% glucose (TSB/Glc) at 37°C with shaking. Following incubation, stationary phase cells were diluted 1:100 in 50% TSB (TSB diluted 1:1 with sodium phosphate buffer at pH 7.4) added with 0.25% Glc and incubated for 24 h at 37°C in polypropylene tubes in a final volume of 100 µL in the presence of different concentrations of each peptide ranging from 1.25 to 40 µM. MIC was determined as the lowest concentration of peptide at which no bacterial growth was visible. For biofilm assays, the two bacterial strains were grown overnight in TSB/Glc at 37°C with shaking. Following incubation, stationary phase cells were diluted 1:100 in 50% TSB/0.25% Glc. 90 µL of each diluted bacterial suspension was dispensed into flat-bottom polystyrene 96 well microtiter plates (Corning Costar, Corning, Tewksbury, MA, USA) and 10 µL of each peptide solution was added to reach final peptide concentrations ranging from 2.5 to 20 µM. Wells without peptide were set up as positive controls. Plates were incubated at 37°C without shaking for 24h. After incubation, biofilm biomass was assessed by crystal violet (CV) staining as previously described (28). Briefly, biofilms were washed with phosphate buffered saline (PBS), air-dried

for 30 min and added with 0.1% CV solution (wt/vol) (bioMérieux, Italy). Following 15 min of incubation at room temperature, wells were washed, air-dried and CV was extracted with 98% ethanol (Sigma-Aldrich). The absorbance was read at 570 nm in a microplate reader. The assays were performed in triplicate, and the results expressed as mean OD_{570} ± standard error of the mean (SEM).

Hemolytic activity. The hemolytic activity of the peptides was determined using fresh human erythrocytes from healthy donors. Blood was centrifuged and the erythrocytes were washed three times with 0.9% NaCl. Peptides dissolved in water were added to the erythrocyte suspension (5%, v/v), at a final concentration ranging from 0.195 to 50 μM in a final volume of 100 μl. Samples were incubated with agitation at 37°C for 40 min. The release of hemoglobin was monitored by measuring the absorbance (Abs) of the supernatant at 415 nm. Control for zero hemolysis (blank) consisted of erythrocytes suspended in 0.9% NaCl. Hypotonically lysed erythrocytes (in water) were used as a standard for 100% hemolysis. The percentage of hemolysis was calculated using the following equation: % hemolysis = [(Abs sample–Abs blank)/(Abs total lysis–Abs blank)] × 100. The results are the mean of three independent experiments.

Preparation of lipid vesicles. Large unilamellar vesicles (LUV) were used for steady-state fluorescence spectroscopy. In order to gradually increase the negative surface charge of the vesicles, we employed the zwitterionic lipid PC and the negatively charged PG at different molar ratios, namely, 0%, 25%, 50% and 75% PG. Weighted amounts of the two lipids were dissolved in chloroform/methanol solution (1/1 vol/vol). The organic solvent was then evaporated under a gentle stream of nitrogen, followed by overnight vacuum pumping to remove any residual. The resulting lipid film was hydrated with 10 mM phosphate buffer (PB) alone or with additional 150 mM NaCl (PBS), at pH 7.4. Multi-lamellar vesicles (MLV) were formed by vortexing for 5x1 min, followed by 5 freeze-thaw cycles. Afterwards, LUV

were prepared with the extrusion method, i.e. by passing the MLV dispersion 11 times through two different pore-size polycarbonate filters (Whatman, Avanti Polar Lipids, Inc.), namely 400 and 100 nm, using the Avanti Polar Lipids mini-extruder.

Fluorescence spectroscopy. Binding investigations were performed by steady-state fluorescence spectroscopy with a LS55 Luminescence Spectrometer (Perkin-Elmer, Waltham, MA, USA) equipped with a thermostatic cuvette holder. LUV at different PC/PG molar ratios were prepared as described hereinbefore. Peptide was added at a final concentration of 1 μM to the buffered vesicles dispersion. Proper aliquots of the LUV stock solution were diluted, in order to obtain different lipid/peptide molar ratios in the 0-500 range. For each peptide, this titration was performed either with PB or PBS (thus, in the absence or in the presence of 150 mM NaCl). Tryptophan fluorescence was measured at 27°C (i.e. well above lipids' phase transition temperature of -2°C), by recording the emission spectrum between 300 and 450 nm. The excitation wavelength was set at 280 nm. Beam entry and exit slit width were set at 5 nm. Each LUV dispersion before peptide addition was used to perform automatic spectrum background subtraction.

Statistical analysis. Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. A p value <0.05 was considered statistically significant.

RESULTS

Antibacterial activity and hemolysis. The antibacterial activity of the selected peptides was first analyzed on two representative strains of Gram-negative and Gram-positive bacteria, i.e. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, respectively, by the microbroth dilution assay to determine the corresponding MIC. As indicated in Table 1, the linear form of the designed analog, lin-SB056-1, resulted to have from 4 to 8-fold lower MIC than the linear

wild-type peptide lin-SB056, either when tested in standard MHB or in MHB supplemented with salt against *E. coli* and in standard MHB when tested against *S. aureus*, while MIC for *S. aureus* in MHB + NaCl was >100 μM for both peptides. This suggests a significantly higher activity, in most cases, of lin-SB056-1 in inhibiting bacterial growth than the parental peptide, whose effectiveness against *E. coli* was prevented by the addition of salt to the culture medium. Interestingly, when the wild-type peptide was synthesized in its dimeric form, the antibacterial potency against *E. coli* in MHB was found to increase by approximately 8-fold, as indicated by the corresponding MIC values (25 μM for the linear form *versus* 3.125 μM of the dimer). This difference was even higher when the two peptides were tested in MHB supplemented with NaCl. A similar trend was shown also against *S. aureus*. Differently, the dimerization of SB056-1 did not influence the MIC of this peptide against both bacterial strains in MHB. However, the addition of salt to the culture medium enhanced by 2-fold the antibacterial activity of the SB056-1 dimer against both strains, while it had the opposite effect in the case of wild-type den-SB056. Importantly, as reported in Table 1, the dimeric form of the analog was found to display the lowest MICs against both bacterial strains and culture conditions (with the exception of *E. coli* in MHB), suggesting that this peptide has an overall better antibacterial activity than the other isoforms either in the absence or in the presence of salt.

Table 2 reports the measured lytic activity of linear and dendrimeric peptides against human red blood cells. In the case of linear, monomeric forms, while the hemolytic activity of the original lin-SB056 was very low at any tested concentration (max 4% at 50 μM), sequence regularization led to a slight increase of cytotoxicity for lin-SB056-lin at high concentrations (max 11% at 50 μM). For both peptides, low hemolytic activity was maintained at peptide concentrations close to the MIC values for both *E. coli* and *S. aureus* (Table 1, in MHB). In the case of dendrimeric den-SB056 and den-SB056-1, haemolytic activity was generally more

elevated than the relevant monomeric form, at any peptide concentration. This effect is likely due to the higher local concentration of bioactive units. However, also for these peptides, at peptide concentrations close to the MIC values for *E. coli* and *S. aureus*, percentage of hemolysis was relatively low, ranging between 5 and 20%. Intriguingly, up to 12.5 μM wild-type den-SB056 was constantly less cytotoxic than the optimized analog den-SB056-1 but haemolytic activity of the former increased rapidly at higher concentrations while that of the analog remained more stable (Table 2).

Bactericidal activity and killing kinetics. To investigate the bactericidal activity of both linear and dendrimeric peptides, we first determined their MBC (Table 3). Remarkably, both dendrimeric isoforms were found to cause 99.9 % killing of the bacterial population at a concentration equal to the MIC. The only exception was given by den-SB056 against *S. aureus*, as a peptide concentration 2-fold higher the MIC was needed to cause 3 $\log_{10}$ reduction in the percentage of living bacteria. In comparison, a peptide concentration 2 to 4-fold higher the MIC was necessary for the achievement of a bactericidal activity by the linear form of both peptides. Next, studies on the killing kinetics of the peptides at their MBC and MBC/2 were performed against the two selected bacterial strains. As reported in Fig. 2, while both linear peptides were able to induce 99.9 % killing of *E. coli* cells within 30-90 min when used at their MBC (Fig. 2, A), a much faster killing rate was demonstrated by the corresponding dendrimeric forms, which were able to provoke the almost complete mortality of the bacterial population within 5 min (Fig. 2, panel B). When the peptides were analyzed against *S. aureus* at their MBC, both lin-SB056-1 and dendrimeric forms were found to cause 99.9% bacterial killing, after 15-30 min (Fig. 2, C-D). Note that when both dendrimer peptides were used at their MBC/2 (Fig. 2, B-C, empty symbols), a reduction in the number of bacterial cells between 97% ($3*10^4$ CFU/ml) and 99.9% (10^3 CFU/ml) was however observed in 90 min. In particular, den-SB056-1 caused 99.7% killing of *E. coli* cells in 15 min (Fig. 2,

B). Interestingly, by comparing the killing kinetics of the dendrimers at the same molar concentration (i.e. 3.125 μM, corresponding to MBC for den-SB056 and MBC/2 for den-SB056-1, as indicated in Table 3), the activity of den-SB056 towards the Gram-negative bacterium *E. coli* resulted to be higher than the dimeric analog, whereas an opposite behaviour was noted against the Gram-positive *S. aureus* (when the two dendrimers were used at 12.5 μM, corresponding to MBC/2 for dim-SB056 and MBC for den-SB056-1).

Antibiofilm activity of linear and dendrimeric peptides. Prior to the assessment of antibiofilm activity, concentrations of dendrimeric and linear peptides able to inhibit growth of planktonic bacteria were determined in biofilm-like conditions against two reference strains of *S. epidermidis* (ATCC 35984) and *P. aeruginosa* (ATCC 27853) (Table 4). Such strains are known to be strong biofilm-producers and represent two bacterial species often involved in biofilm-associated infections. When the peptides were tested under these conditions against planktonic cells, an overall increment of their MIC values was observed as compared to standard conditions, against both species (data not shown). In biofilm-like conditions den-SB056 and lin-SB056-1 exhibited MIC values lower than den-SB056-1 and lin-SB056 respectively, against *S. epidermidis*. MIC values higher than 40 μM were recorded for all four peptides against *P. aeruginosa* (Table 4). The ability of the peptides to inhibit biofilm formation was then evaluated against the same strains. The inhibitory effect was assessed as reduction of biofilm biomass by CV staining, after incubation of the strains for 24 h with different concentrations of the peptides. As shown in Fig. 3, A, all the peptides, with the exception of lin-SB056, were able to reduce the biofilm biomass of *S. epidermidis* of more than 50% as compared to the control biofilm at concentrations of 5-10 μM. den-SB056 and lin-SB056-1 exhibited the strongest antibiofilm activity, causing a reduction of approximately 98% of the biofilm biomass, when assayed at 10 μM. When the peptides were tested against *P. aeruginosa* (Fig. 3, B), a poor antibiofilm activity of den-SB056-1 was observed, while

den-SB056, lin-SB056 and lin-SB056-1 caused a marked reduction of biofilm biomass but only at the concentration of 20 μM. Similarly to what observed with *S. epidermidis*, den-SB056 and lin-SB056-1 demonstrated the highest activity against sessile *P. aeruginosa* cells, causing a reduction of the biofilm biomass of approximately 90% as compared to the un-treated control, at the active concentration of 20 μM.

Fluorescence spectroscopy: peptide-lipids binding. Upon binding, peptide's tryptophan moves from the buffered water to the more hydrophobic environment characterizing the lipid bilayer of LUV. Typically, this leads to a blue shift and an increase in the quantum yield of tryptophan fluorescence (29, 30). However, fluorescence intensity depends on several different factors, hard to be quantitatively taken into account. For instance, after the binding, if peptide oligomerization occurs, tryptophan residues could result in close proximity with each other leading to fluorescence self-quenching. Another source of quenching might be the interaction between tryptophan and the positively charged peptide residues (31), like the tryptophan flanking lysines in the peptides under investigation. Moreover, quenching could also be due to the charged head group of PG interacting with tryptophan π-orbitals (29). All of these contributions strictly depend upon the specific secondary structure adopted by the peptide, possible oligomerization and peptide orientation with respect to the bilayer. Insertion depth is also fundamental, as well as the specific position of the tryptophan along the peptide sequence. In addition, the bilayer composition should exert an important influence on the observed fluorescence intensity. Thus, even if in principle intensity as a function of the lipid/peptide ([L]/[P]) ratio could be related to peptide binding constant (30), no attempt was made in the present work to quantitatively estimate the latter, since accurate structural information about the vesicles-bound state of SB056 peptides is still lacking. Nevertheless, tryptophan blue shift is able to provide important qualitative information about the relative affinity of different analogues for the same membrane model. Similarly, the relative binding

affinity of a given peptide for differently charged membrane models can be evaluated. Emission wavelength (λ) decreases with increasing [L]/[P] until saturation is usually reached (29, 30). Although the maximum difference ($\Delta\lambda_{max}$) between saturation and starting λ (in the absence of lipids) might depend upon several factors similarly to fluorescence intensity, the higher the peptide affinity for the lipid bilayer, the lower the saturation [L]/[P] value. In particular, we investigated interaction of the four SB056 analogues with model membranes with increasing PC/PG molar ratio, since it is known that eukaryotic membranes are characterized by a very low content of negatively charged lipids, while this is remarkably higher in bacterial plasma membranes (4, 32). In addition, Gram-negative bacteria typically have a content of negatively charged lipids around 30%, while it is 70% or more in Gram-positive ones (33, 34). Moreover, as already pointed out for MIC assays, we investigated the effect of physiological electrolytes concentration on the peptide-lipids interaction by performing the same experiments in 10 mM PB either in the absence or in the presence of 150 mM NaCl. Fig. 4 shows all results; the absolute value of Δλ is plotted as a function of the [L]/[P] molar ratio. In all investigated cases, peptide-lipids affinity increased with increasing the PG content of vesicles. At low ionic strength (thus, in the absence of NaCl), lin-SB056 showed a remarkable affinity starting from 50% PG (Fig. 4, A), while its dendrimeric counterpart den-SB056 from 25% PG (Fig. 4, B). This might be simply explained with the dendrimeric peptide having twice the positive charge per mole. Neither the linear nor the dendrimeric peptide showed a significant affinity for the 100% PC liposomes as expected. The modified dendrimeric peptide den-SB056-1 (Fig. 4, D) showed a comparable trend, but resulted to have a not-negligible, although low, affinity for the 100% PC membrane. Quite surprisingly, lin-SB056-1 was the peptide with the highest affinity for all the investigated vesicles (Fig. 4, C), as shown by the tryptophan fluorescence shift going to saturation faster than observed for all the other analogues. The interaction was absolutely remarkable even for

the 100% PC bilayer. As expected, electrolytes addition decreased affinity of all the peptides (Fig. 4, A-D, black lines), confirming the fundamental role of electrostatics in the very first step of peptide action, which is the membrane binding. Nevertheless, similarly to what was observed for antimicrobial activity, while salt effect was dramatic for both linear peptides, dendrimeric ones were far less affected. However, by comparing the two dendrimeric peptides, it is interesting to note that den-SB056-1 retains membrane affinity in the presence of salt to a higher extent than den-SB056, despite they bear exactly the same net positive charge.

DISCUSSION

In this study, analogs of the antimicrobial peptide SB056 have been synthesized and tested for antimicrobial activity in the presence of physiological electrolytes concentrations and against biofilms. Analogs have been designed to display an enhanced amphiphilic profile, so to optimize peptide interaction with target membranes, and the properties of dendrimeric (dimeric) structures compared to linear, monomeric forms (see Fig 1). SB056 is an antimicrobial peptide with innovative design and intriguing properties. The results obtained, confirm this idea. At low ionic strength, den-SB056 resulted to be more active than its linear counterpart, against both Gram-negative and -positive strains (Table 1). Thus, the original idea behind dendrimeric peptides design seems to be confirmed. This increased activity is usually attributed to the higher local concentration of bioactive units due to the branched dendrimeric structure, besides the greater stability against peptidases and proteases (15, 35). In the present work, the SB056-1 analogue has been synthesized by exchanging the position of the first two amino acid residues. SB056 sequence is characterized by an alternate pattern of hydrophobic and polar/charged residues, suggesting an amphipathic β-type folding upon interaction with lipid membranes (21), already confirmed for the linear analogues (22). Such

an alternate pattern is observed throughout the sequence but the first three residues (WKK). This discrepancy has been removed in the SB056-1 analogue (KWK), based on the hypothesis that the higher is the intrinsic amphipathicity of the sequence, the higher the structural order and stability of the β-sheet oligomers on the membrane surface, the higher the antimicrobial activity (21, 22). Consistently, lin-SB056-1, showed an improved antimicrobial activity against all the strains investigated when compared to the lin-SB056, resulting to be sometimes comparable to that of den-SB056. Also, the dendrimeric den-SB056-1 showed similar MIC values to its linear counterpart, further supporting the hypothesized sequence optimization which makes it possible to attain a more ordered amphipathic structure.

The inhibition of activity in presence of physiological salts concentration is still a severe limitation to AMPs' applicability as drugs *in vivo* (9, 10, 36). As usually observed for linear peptides, by increasing the ionic strength of the environment, lin-SB056 and to a lesser extent also lin-SB056-1 showed a significant decrease of the antimicrobial activity. This is likely due to the charge shielding produced by counterions cloud on both the peptide and the membrane surface, thus reducing their long-range electrostatic interaction. On the other hand, both the dendrimeric peptides retained their antimicrobial activity (in some cases it even improved) in the same environment, clearly showing that the dendrimeric structure is somehow able to overcome such electrolytes shielding effect. This might be explained by taking into account the two main features of these dendrimeric peptides. Possibly, the increased positive charge density, due to the two peptide branches, is not as efficiently shielded as for the linear peptides. In addition, despite the reduced electrostatic interaction with the membrane surface in an environment with physiological salt concentration, the hydrophobic tail at the C-term might act as an anchor, stabilizing the peptide bound-state. Thus, the advantages offered by the dendrimeric structure seem to be fundamental, especially when the environment approaches the *in vivo* conditions. The same behavior has been observed for antimicrobial

peptide dendrimers designed around dendronized ornithine (37). Furthermore, both our dendrimeric peptides displayed a much faster microbicidal kinetics with respect to their linear counterparts. Finally, it is interesting to note that, despite a general difference in the content of negatively charged lipids is known between Gram-positive and Gram-negative bacteria (it is significantly higher in the former) (33, 34), the relative antimicrobial activity observed for *S. aureus* is somewhat lower than that seen for the Gram-negative *E. coli*.

Concern regarding biofilm-associated infections is growing rapidly worldwide, as biofilms are inherently tolerant and resistant to antimicrobial therapies. Moreover, they tend to develop on medical device surfaces, further increasing the risk of hospital-acquired infections and of microbial dissemination within the host (38, 39). The need to deploy additional bactericidal means is particularly urgent in this case, and many AMPs have been tested for their ability to target specific features of sessile bacterial communities (40). A dedicated database, BaAMPs (http://www.baamps.it), is available that reports information on the anti-biofilm activity of AMPs in an organized framework (41). In our hands, SB056 and analogs proved to be endowed with interesting anti-biofilm properties (intended as inhibition of biofilm formation). In particular, *S. epidermidis* was the most susceptible strain in sessile form, especially to lin-SB056-1 and the wild-type dendrimer den-SB056. The same peptides showed the best activity among the tested compounds also against *P. aeruginosa* biofilms, although with lower overall efficiency with respect to *S. epidermidis*. Interestingly, the antibiofilm effect of all the peptides was observed at concentrations equal or lower than the corresponding MIC values evaluated in biofilm-like conditions (Table 4), suggesting that the reduction in biofilm biomass was attributable, at least in part, to mechanisms other than a direct killing effect. Comparing the wild-type peptides, these experiments confirmed the value of the dendrimeric structure over the linear one, while the optimized structure proved to work at best when in its linear form. Improvement of the β-sheet stability, as said, seems correlated

to the antimicrobial activity. However, if exaggerated, it is possibly detrimental to oligomers dynamics and compromises diffusion and penetration into the biofilm. Overall, it is apparent that the class of molecules presented here displays good potential to be developed as antibiotics to fight infections caused by both Gram-positive and –negative strains, even in biofilm form, but action appears somewhat selective.

Membrane-binding experiments revealed that in all cases peptide affinity increased with increasing the PG content of vesicles. This was not surprising, since electrostatics play a major role in peptide binding and is thought to be responsible for peptide selectivity for bacterial membranes. Indeed, differently from eukaryotic membranes, prokaryotic ones are characterized by a significant content of negatively charged lipids, thus strongly attracting positively charged antimicrobial peptides (4, 10). However, the linear analog lin-SB056-1 was the peptide with the highest binding affinity for all the investigated compositions, even for the zwitterionic 100% PC bilayer. This is not compatible with the interaction being driven only by electrostatics, since the linear peptide has half the positive charge per mole with respect to the dendrimeric one. In addition, the sequence modification consisted simply in exchanging the first two residues, thus leaving the total charge unaffected. Nevertheless, lin-SB056 and lin-SB056-1 showed remarkable differences in their relative affinity for the same membrane model, the lowest and the highest, respectively, among the four peptides investigated. It is very interesting to note how these results are in good agreement with the MICs estimated in the absence of added NaCl (Table 1): the wild-type linear lin-SB056 showed, in general, the lowest membrane affinity and, indeed, it was the less active, while the analog lin-SB056-1 showed the highest affinity and, correspondingly, it showed an excellent antimicrobial potency. These results show that, despite electrostatics play a major role, charge distribution along the sequence may significantly affect membrane interaction. A strong peptide-membrane electrostatic interaction is fundamental to bring the peptide from the aqueous

environment to membrane proximity. However, in our case, an optimal distribution of the charged/polar residues along the sequence entails an optimal distribution of the hydrophobic residues too, explaining the higher membrane affinity observed in the case of lin-SB056-1 even to 100% PC vesicles. By comparing the two dendrimeric peptides, it is interesting to note that den-SB056-1 retains binding affinity with increasing the ionic strength more than den-SB056, despite they bear exactly the same net positive charge, further showing that distribution of charged/polar and hydrophobic residues along the sequence is very important as peptide behavior determinant.

Conclusion. A schematic summary of the antimicrobial activities and the affinity for lipid membranes, either in the presence or in the absence of salt at physiological concentrations, for the four peptides is presented in Fig. 5. A lot of information is still lacking on the activity of these novel molecules, such as an in-depth structural investigation either in free or membrane-bound form, which is currently underway in our labs. Nevertheless, our sequence optimization based on available structural data resulted to be really effective and the dendrimeric branched arrangement of the functional units showed their advantages, with dendrimeric peptides – but not their linear counterparts – having their membrane-binding properties and antimicrobial activity preserved even at physiological electrolytes concentration. Despite these positive findings, cytotoxicity studies indicate however that the optimization process is not complete, and further efforts must be devoted to obtain dendrimeric peptides with lower hemolytic activity, likely by fine-tuning their affinity for zwitterionic membranes. Rational modification of AMPs, driven by the study of structure-function relationships, is a powerful tool for the identification of therapeutically useful antibiotics.

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CONFLICT OF INTEREST

AG and GP are minor shareholders of Spider Biotech S.r.l.

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Figure legends

Figure 1 Structure of SB056 and analogues. In (A) a schematic representation of the characteristic dendrimeric structure of the SB056 peptide. In (B) the amphipathic pattern of SB056 and SB056-1 sequences are presented together with their hydropathicity plot. In SB056-1, the position of Trp-1 and Lys-2 is inverted. Linear peptides lin-SB056 and lin-SB056-1 comprise only the relevant, monomeric peptide unit. Blue and yellow circles are used to indicate hydrophilic and hydrophobic residues, respectively. The hydropathicity plot was calculated using the method of Kyte and Doolittle [42] with a window of three residues and a weight of 30% at the window edges. The values are normalized between 0 and 1. The higher the value the more hydrophobic is the residue.

Figure 2 Killing kinetic of SB056 (square) and SB056-1 (circle) peptides on *E. coli* ATCC 25922 (A and B) and *S. aureus* ATCC 25923 (C and D). Bacteria (1x106 CFU/ml) were incubated at MBC concentrations (filled symbols) and at MBC/2 concentrations (empty symbols) (see Table 3) in MHB at 37°C. The control (triangle) is given by bacteria without peptide. Data point represent the mean of triplicate samples ± SD. The dotted lines indicate 99.9% bacterial killing.

Figure 3 Inhibitory effect of dendrimeric and linear peptides on biofilm formation of *S. epidermidis* ATCC 35984 and *P. aeruginosa* ATCC 27853. Bacterial cells of *S. epidermidis* (A) and *P. aeruginosa* (B) were incubated at 37°C in a 96-well plate in the presence of different concentrations of the peptides for 24h. Cultures without peptides were used as a positive control (CTRL). Biofilm biomass was determined by crystal violet staining and expressed as OD570. Solid lines and dotted lines represent respectively 50% and 90%

reduction of biofilm biomass, as compared to the control biofilms. Data represent the mean ± the SEM of at least three independent experiments. Stars indicate statistically significant differences between the positive control and the biofilms obtained in the presence of the different peptides. **p<0.01; *** p<0.001 (one way ANOVA followed by Tukey–Kramer multiple comparison test).

Figure 4 Peptide interaction with model membranes. The blue shift of tryptophan fluorescence emission $|\Delta\lambda|$ is plotted as a function of [L]/[P] ratio for the four SB056 analogues in the presence of differently charged PC/PG LUVs. The lower the [L]/[P] ratio needed to reach saturation, the higher the peptide affinity. Measurements were carried out in 10 mM PBS either in the absence (red lines) or in the presence (black lines) of additional 150 mM NaCl.

Figure 5 Schematic summary of the antimicrobial activity (A and B) and the affinity for lipid membranes (C and D), either in the presence or in the absence of salt at physiological concentrations, for the four peptides. Each peptide is represented by a colour and differently sized segments of the graph reflect differences in the investigated activity. (A) includes anti-biofilm activity, investigated at low ionic strength. As depicted, the wild-type lin-SB056 resulted to be the worst peptide independently of the environment investigated, while its dendrimeric counterpart, den-SB056, did not show any drastic change going from low to high ionic strength environment, revealing a good, constant activity. The optimized analogue lin-SB056-1 revealed to be the best peptide at low ionic strength, but these activities decreased as the electrolytes concentration approached the physiological one. On the contrary, den-SB056-1 increased its activity moving from low to high ionic strength. See main text for further details and discussion.

Tables

Table 1 Minimal Inhibitory Concentration (MIC) values, expressed in μM, of SB056 and its analog SB056-1 in their linear and dendrimeric forms against the Gram-negative *E. coli* ATCC 25922 and the Gram-positive *S. aureus* ATCC 25923 in MHB or MHB supplemented with 150 mM NaCl.

	E. coli ATCC 25922		*S. aureus* ATCC 25923	
Peptide	MHB	MHB + NaCl	MHB	MHB + NaCl
lin-SB056	25	>100	>100	>100
lin-SB056-1	6.25	12.5	12.5	>100
den-SB056	3.125	6.25	12.5	25
den-SB056-1	6.25	3.125	12.5	6.25

Table 2 Hemolytic activities of SB056 and its analog SB056-1 in their linear and dendrimeric forms, expressed in μM.

	% Hemolysis								
Peptide	**Peptide concentration (μM)**								
	50	**25**	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**0.39**	**0.195**
lin-SB056	4	2	2	2	2	0	ND	ND	ND
lin-SB056-1	11	11	6	6	2	2	2	ND	ND
den-SB056	62	36	20	14	5	5	4	ND	ND
den-SB056-1	34	24	21	16	15	15	11	8	4

ND. Not determined. The results are the mean of three independent experiments. Data for lin-SB056 and lin-SB056-1 taken from [22].

Table 3 Minimal bactericidal concentrations (MBC), expressed in μM, of SB056 and its analog SB056-1 in their linear and dendrimeric forms against the Gram-negative *E. coli* ATCC 25922 and the Gram-positive *S. aureus* ATCC 25923 strains after 90 min incubation at 37°C in MHB medium.

Peptide	*E. coli* ATCC 25922	*S. aureus* ATCC 25923
lin-SB056	100	>100
lin-SB056-1	12.5	50
den-SB056	3.125	25
den-SB056-1	6.25	12.5

Table 4 Minimal Inhibitory Concentration (MIC) values, expressed in μM, of SB056 and its analog SB056-1 in their linear and dendrimeric forms against planktonic *S. epidermidis* ATCC 35984 and *P. aeruginosa* ATCC 27853 in biofilm-like conditions.

Peptide	*S. epidermidis* ATCC 35984	*P. aeruginosa* ATCC 27853
lin-SB056	>40	>40
lin-SB056-1	10	>40
den-SB056	40	>40
den-SB056-1	>40	>40

MIC values obtained in biofilm-like conditions (stationary phase cells in 50% TSB/Glc 0.25%).

Figure 1

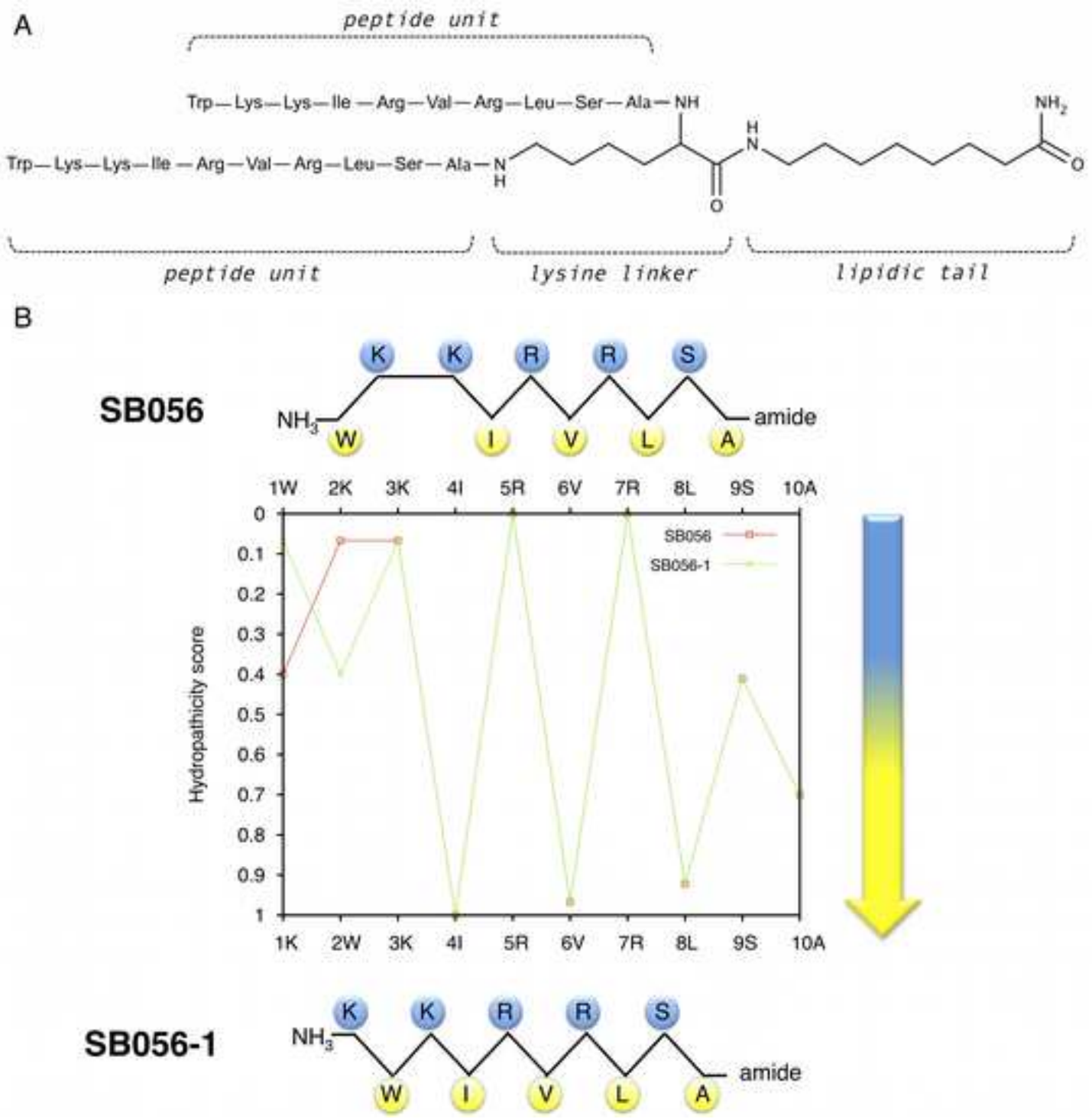

Figure 2

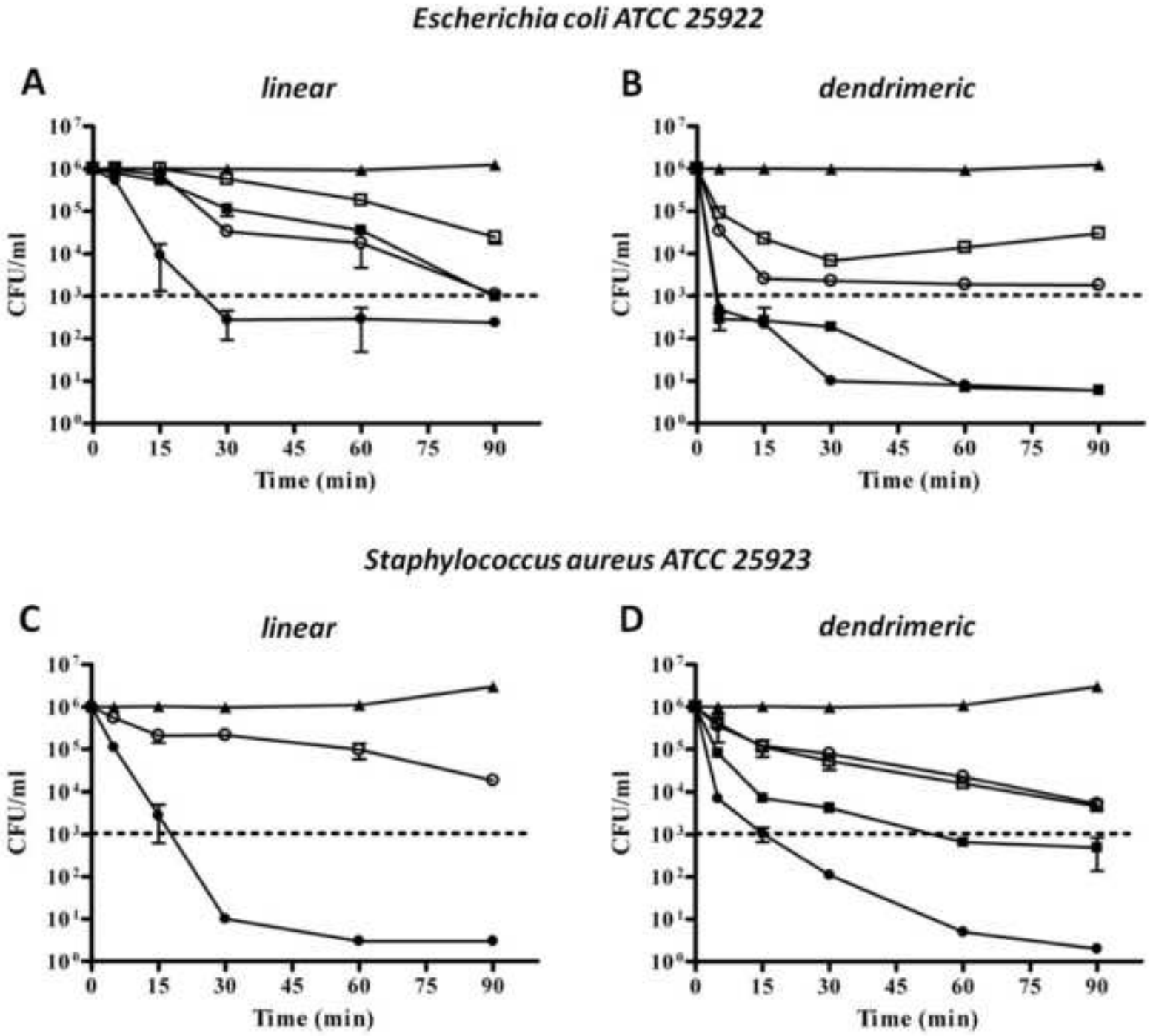

Figure 3

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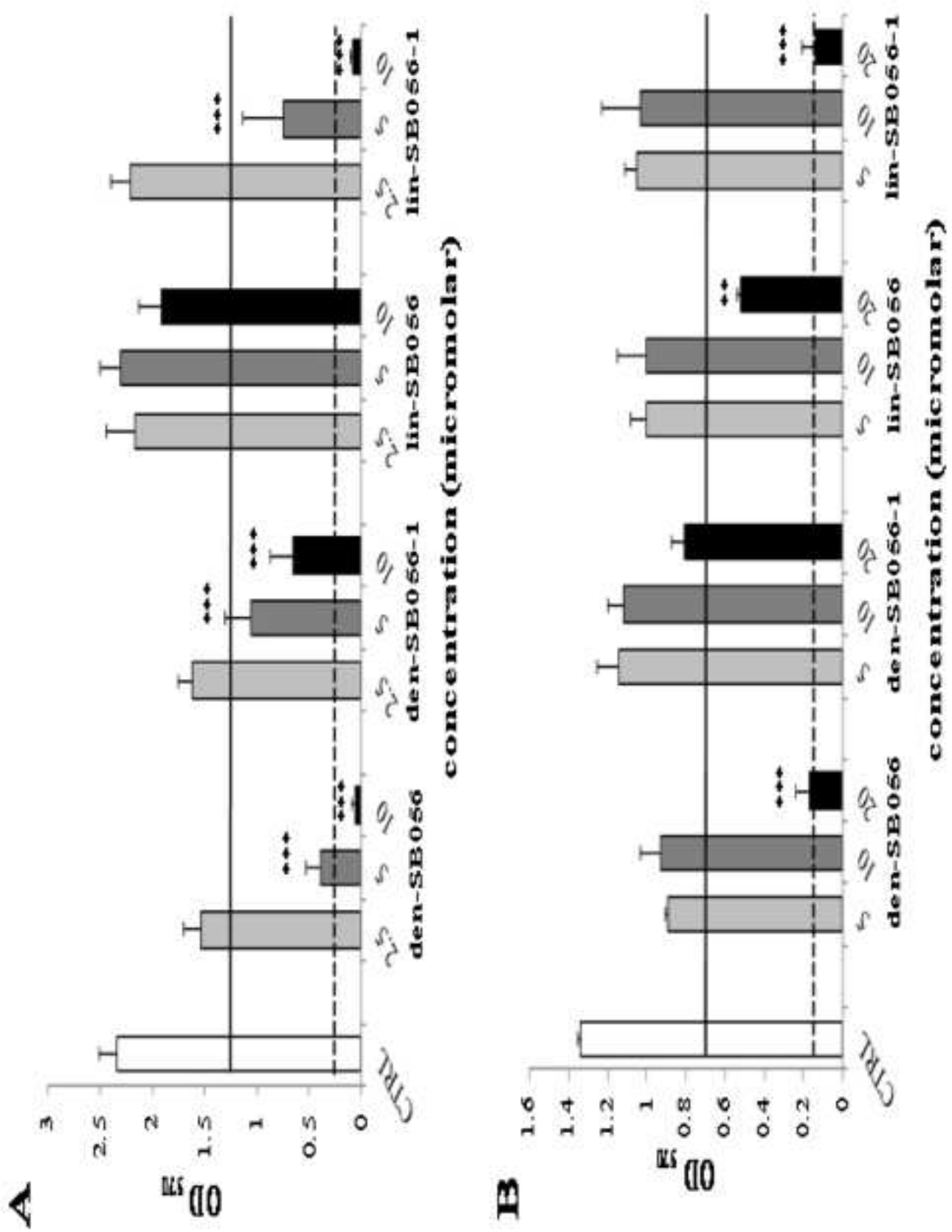

Figure 4

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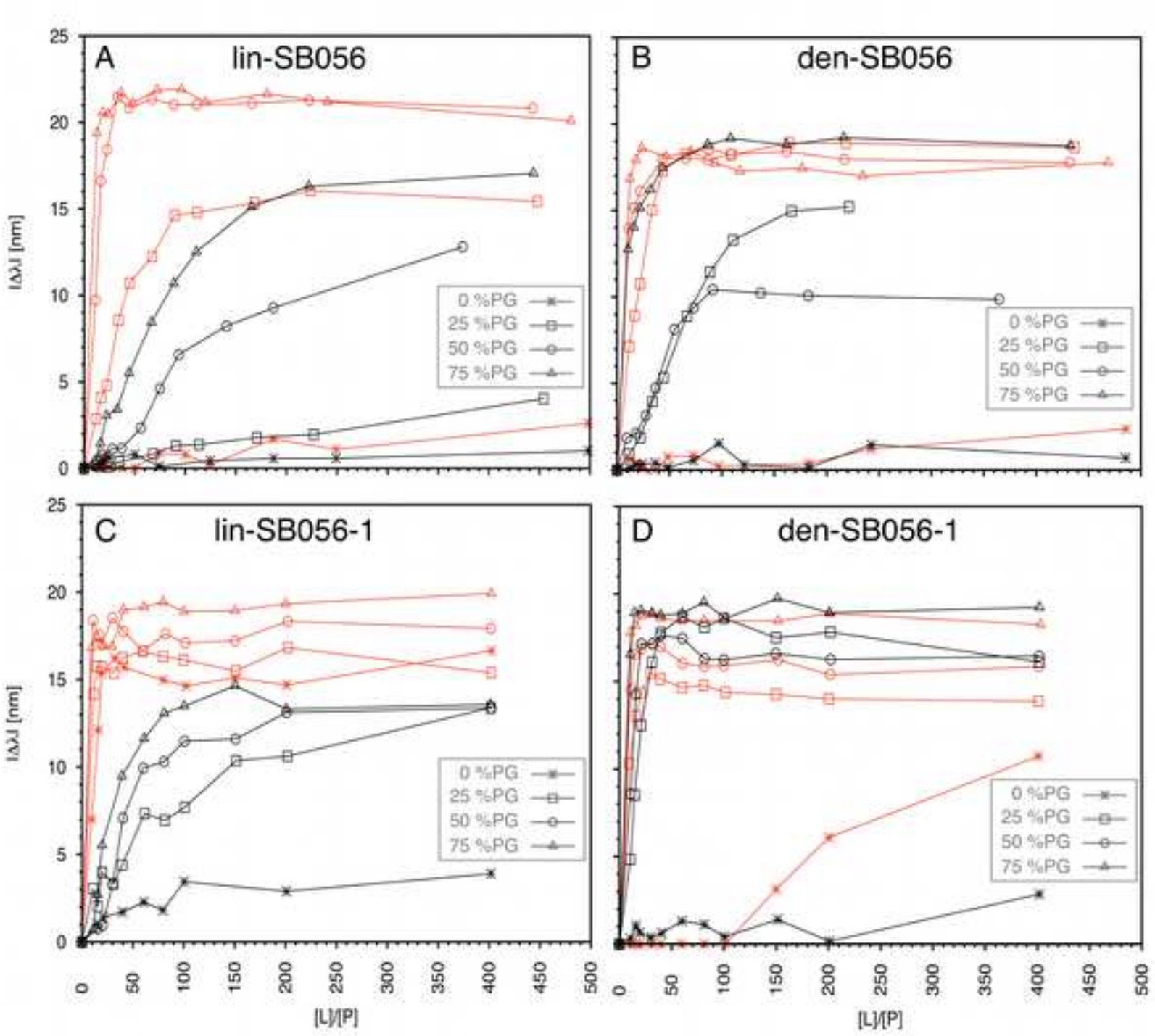

Figure 5

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