Characterization, mechanism of action and optimization of activity of a novel peptide-peptoid hybrid against bacterial pathogens involved in canine skin infections

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Integumentary infections like pyoderma represent the main reason for antimicrobial prescription in dogs. Staphylococcus pseudintermedius and Pseudomonas aeruginosa are frequently identified in these infections, and both bacteria are challenging to combat due to resistance. To avoid use of important human antibiotics for treatment of animal infections there is a pressing need for novel narrow-spectrum antimicrobial agents in veterinary medicine. Herein, we characterize the in vitro activity of the novel peptide-peptoid hybrid B1 against canine isolates of S. pseudintermedius and P. aeruginosa. B1 showed potent minimum inhibitory concentrations (MICs) against canine S. pseudintermedius and P. aeruginosa isolates as well rapid killing kinetics. B1 was found to disrupt the membrane integrity and affect cell-wall synthesis in methicillin-resistant S. pseudintermedius (MRSP). We generated 28 analogues of B1, showing comparable haemolysis and MICs against MRSP and P. aeruginosa. The most active analogues (23, 26) and B1 were tested against a collection of clinical isolates from canine, of which only B1 showed potent activity. Our best compound 26, displayed activity against P. aeruginosa and S. pseudintermedius, but not the closely related S. aureus. This work shows that design of target-specific veterinary antimicrobial agents is possible, even species within a genus, and deserves further exploration.

Staphylococcus pseudintermedius is a commensal bacterium colonizing dog skin and mucosal sites1, and it is the predominant cause of canine pyoderma and otitis externa2. These common infections represent the main reason for antimicrobial prescription in dogs3. Over the last decade, methicillin-resistant S. pseudintermedius (MRSP) has been reported worldwide4, including sporadic infections in humans in contact with dogs5,6. Pseudomonas aeruginosa is another pathogen frequently involved in canine integumentary infections, in particular otitis externa7. P. aeruginosa is resistant to most antibiotics used in veterinary medicine. The presence of this pathogen and the increasing frequency of multiresistant MRSP8, make treatment of dogs with integumentary infections difficult or even impossible in some cases9. In light of the few treatment options available against these pathogens, new therapeutic agents are needed, preferably drugs restricted to veterinary use and with a narrow spectrum10. This would limit their impact on the commensal microbiota11.

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In recent years, antimicrobial peptides (AMPs) have attracted considerable interest as alternative anti-infectives\textsuperscript{12}. AMPs are present in all multicellular organisms as part of their innate immune systems\textsuperscript{13}. They show selective toxicity towards bacteria, rapid killing, broad-spectrum antimicrobial activity, and are active at micromolar concentrations or lower\textsuperscript{14}. Furthermore, they possess immunomodulatory properties such as leukocyte recruitment and suppression of harmful inflammation\textsuperscript{15}. Most AMPs exhibit their antimicrobial activity by disrupting the bacterial cell membrane; however, intracellular targets have also been reported\textsuperscript{16}.

The main drawbacks of AMPs as therapeutics are toxicity and susceptibility to proteases\textsuperscript{17}. Traditionally, these problems are overcome by chemical modification, such as cyclization or design of peptidomimetics, which are stable to proteases\textsuperscript{18}. We and others have previously investigated antimicrobial N-alkylglycines (peptoids)\textsuperscript{19}, β-peptoids (N-alkyl-β-alanine oligomers)\textsuperscript{20}, β-peptides\textsuperscript{21}, lysine-based α-peptide/β-peptoids\textsuperscript{22}, and α/γ N-Acylated-N-aminoethylpeptides (AApeptides)\textsuperscript{23}. Some studies have reported activity of peptides and peptidomimetics against veterinary pathogens\textsuperscript{24}. However, only a few of them aimed at the design and optimization of AMPs with activity against \textit{S. pseudintermedius}\textsuperscript{25–28} and canine strains of \textit{P. aeruginosa}\textsuperscript{29}.

The peptide-peptoid hybrid B1 (Fig. 1) has been previously identified and described as active against one clinical isolate of \textit{S. pseudintermedius} and \textit{P. aeruginosa}, as well as resistant to proteolytic degradation in conditions resembling \textit{in vivo} metabolism\textsuperscript{30}. The aim of the present study was to investigate the antimicrobial activity of B1 against a large collection of \textit{S. pseudintermedius} and \textit{P. aeruginosa} isolates from canine infections, determine time-kill kinetics and probe the mechanism of action against \textit{S. pseudintermedius}. Furthermore, we designed and tested 28 analogues of B1 and selected the peptides 23 and 26 (Fig. 1) for their improved selectivity against \textit{S. pseudintermedius} compared to B1 while retaining comparable activity against \textit{P. aeruginosa}. Next, we aimed to get insight into the antibacterial activity of B1, 23 and 26 against other bacterial species causing infections in dogs. Finally, we closely investigated the selectivity of compounds 23 and 26 against \textit{S. aureus}. This study demonstrates that design of peptide-based antimicrobials which target specific veterinary bacterial species is possible.

**Results**

**Antimicrobial activity and killing kinetics.** Compound B1 was identified from a combinatorial library. Further evaluation of B1’s antimicrobial activity against a panel of 57 \textit{S. pseudintermedius} isolates from canine infections revealed consistently low MICs (2–4 µg/mL), irrespective of methicillin resistance (Fig. S5, Supplementary Information). Similar MICs (4–8 µg/mL) were observed for the three \textit{S. aureus} isolates tested (data not shown). Furthermore, MICs of B1 against a panel of 50 \textit{P. aeruginosa} isolates ranged from 8–16 µg/mL.

The time-kill kinetic assay showed complete killing of the tested MRSP strain E104 (MIC = 8–16 µg/mL) in 2 h at 2 × MIC, and in 1 h at 4 × MIC, revealing a rapid concentration-dependent effect (Fig. 2a). A similar but
slightly inferior effect was detected against the clinical strain *P. aeruginosa* 26314, which was killed at 4x MIC in 2 h and at 2x MIC in 24 h (Fig. 2b). Lower concentrations (1x MIC) of B1 did not eliminate the *Pseudomonas* strain but resulted in delayed re-growth (Fig. 2b).

**Mode of action of B1 against *S. pseudintermedius* E104.** The mode of action of B1 was investigated by studying the effect of sub-inhibitory concentrations of B1 on membrane potential and macromolecule synthesis rate of the MRSP strain E104. The well-characterized antimicrobial nisin was used as a control. Growth curve analysis of B1 at 3 µg/ml showed cell lysis, as indicated by a decrease of OD over time (Fig. S6 Supplemental Material). However, at lower concentrations, B1 resulted in only minor growth inhibition. This is in contrast with nisin, which in addition to cell lysis at MIC concentration (5 µg/ml), significantly retarded growth, also at sub-inhibitory concentrations.

In order to determine if the lysis of MRSP E104 by B1 was due to the inhibition of cell wall biosynthesis, the synthesis rate of cell wall macromolecules was studied at sub-inhibitory concentrations (1.5 µg/mL). In addition, since cationic AMPs have been reported to interact with DNA due to their positive charge and hydrophobicity, the DNA replication was also measured. B1 resulted in a 20% reduction in cell wall synthesis without affecting DNA replication (Fig. 3). Exposure to nisin resulted in 50% inhibition of DNA synthesis (Fig. 3a) and 60% inhibition of cell wall synthesis (Fig. 3b).

To test the immediate effect of a sub-inhibitory concentration of B1 (1.5 µg/mL) on membrane potential, proton motive force (PMF) was measured using DiSC3(5) (3,3’-Dipropylthiadicarbocyanine Iodide), a fluorescent probe that concentrates in energized membranes and is released in the environment surrounding a cell upon membrane depolarization, thus increasing the intensity of its emission. As expected, no fluorescence increment was observed after nisin treatment (Fig. 4a,b). On the contrary, B1 caused significant membrane depolarization, as indicated by an increased DiSC3(5) fluorescence emission (Fig. 5a,b). Furthermore, the energy dissipation effect of B1 on the cell at the lysis concentration (1x MIC) was studied by flow cytometry analysis. Cell death (Fig. 5c, P2) and injury (P3) upon B1 exposure was indicated by the high ratio of propidium iodide (PI) staining relative to thiazole orange (TO) staining, whereas unexposed control cells were mainly stained with TO (Fig. 5b). Taken together, our studies indicated that B1 causes membrane depolarization and affects cell-wall synthesis but not DNA-synthesis.
Analogues of B1. After characterizing B1 as a lead, we proceeded to generate an ensemble of 28 analogues (Table S1, Supplementary Information) in order to develop compounds with specific activity against S. pseudintermedius and P. aeruginosa, paired with low activity against methicillin-susceptible Staphylococcus aureus (MSSA). Compounds 2–29 are analogues of B1 (Fig. 1A). Compounds 2–5 (Table 1) contain L-Lys and peptoid residues. In compound 2, residue 6 (N-4-methylbenzylglycine) was replaced with N-benzylglycine resembling Phe; in compound 3, residue 4, N-butylglycine, was switched with residue 5, Lys, to generate a hydrophobic and cationic cluster. Compounds 4 and 5 are the reversed compounds of 2 and 3.

Compounds 6–13 contain L-lysine and L-amino acids (Leu, Phe, 1-Nal, 2-Nal) instead of the peptoid residues N-butylglycine, N-1-naphthylmethylglycine and N-4-methylbenzylglycine, respectively. In compound 6, peptoid residues have been replaced with the corresponding amino acids and in 7, residue Leu⁴ and residue Lys⁵ have been switched to obtain a hydrophobic and cationic cluster. Compounds 8 and 9 differ from 6 and 7 for the presence of 2- instead of 1-Nal. Compounds 10–13 are the reverse of 6–9. Compounds 14–21 are analogues of 6–13 in which L-Lys residues have been retained and Leu, 1-Nal, 2-Nal and Phe have been replaced by the corresponding D-stereoisomers.

Based on the data for 1–21, we synthesized a second set of compounds (22–29) maintaining three L-Lys at the N-terminus except for 29. Compounds 22–23 are analogues of 9 in which Phe⁶ and Leu⁴ were replaced by Nle and Tyr, respectively. Compound 23 is an analogue of 7 in which Leu⁴ was replaced with Nle. Compounds 25 and 26 are derived from 15, in which D-Phe⁶ and D-Leu⁴ have been replaced by D-Nle and D-Tyr, respectively. Compounds 27 and 28 contain combinations of substitutions introduced in compounds 22–26. Finally, compound 29 is an analogue of 9 in which Lys residues have been replaced by N-(4-aminobutyl)glycine.

MICs of the analogues were determined against S. pseudintermedius C22963, MSSA (ATCC 29213), and P. aeruginosa 26314. The sequence of each analog, the MICs and hemolysis data against red blood cells are reported in Table 1.
Compounds 2–21 were generally less active than B1 with MICs of 2–16 µg/mL against S. pseudintermedius C22963, 8–64 µg/mL against S. aureus, and 8–>64 µg/mL against P. aeruginosa 26314. Hemolysis at 150 µM for compounds 2–17 ranged from <8–96% while the reverse sequences 18–21 were not hemolytic.

To further improve selectivity and hemolysis, we synthesized a second set of compounds (22–29) maintaining three L-Lys at the N-terminus except for 29. Five of the compounds featuring three Lys (or Lys-like residues, 29) showed 16-fold better activity against the MRSP strain (MIC 2–16 µg/mL) than against the MSSA strain (MIC 32–>64 µg/mL). Furthermore, they showed moderate activity against MRSA USA300 strain FPR3757 (32–>64 µg/mL) (data not shown). In addition, compound 23, 25 and 26 retained the activity level of B1 against P. aeruginosa (8 µg/mL). The haemolytic values of compounds 22–29 ranged from 46 to 64% at 150 µM except for compound 22 (14%). The two most promising analogues (23 and 26) were selected for further studies due to (i) the low MICs observed in MRSP and P. aeruginosa, (ii) the relatively higher antimicrobial effect against S. pseudintermedius compared to S. aureus, and (iii) a moderate to low-level of hemolysis EC_{50} of 104 µM and 63 µM, respectively (Table 1).

**Time-kill experiments of 23 and 26.** Time-kill experiments showed that 26 (Fig. 6). was superior to both B1 and 23 (Fig. 7a) and displayed complete killing of MRSP C22963 within 1 h at only 0.5X MIC Similarly to B1, these two analogues exhibited complete and concentration-dependent killing of the MRSP strain within 2 h at all tested concentrations or above the MIC. Surprisingly, 23 was not able to completely kill P. aeruginosa, even at the highest tested concentration (8X MIC) (Fig. 7b). We also intended to do time-kill curves for 26 against P. aeruginosa (MIC = 16 µg/mL). To our disappointment, 26 precipitated after 15 min in the media, and it was not possible to get reproducible results.

**Antimicrobial activity of B1, 23 and 26 against a selection of canine pathogens.** In order to get further insight into the antimicrobial spectrum of B1, 23 and 26, we tested MICs against a collection of clinical isolates representing other canine pathogens (Corynebacterium auriscanis, Enterococcus faecalis, Enterococcus
faecium, Streptococcus canis, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumonia, Pasteurella canis, Proteus mirabilis) (Table 2). All three compounds showed activity against Gram-positive bacteria with MICs ranging between 2–16 µg/mL, except for Enterococcus (>64 mg/mL). However, B1, 23 and 26 were substantially less active against the Gram-negative bacteria with most of the MICs in the range of 32–>64 µg/mL, P. aeruginosa and Pasteurella canis being the only exceptions. For the latter species, the effect varied consistently between B1, 23 and 26 (8 µg/mL, >64 µg/mL and >64 µg/mL, respectively).
Selectivity of 23 and 26 between *S. pseudintermedius* and *S. aureus*. Finally, we tested the selectivity of 23 and 26 against clinical isolates of *S. pseudintermedius* (n = 10) vs *S. aureus* (n = 10) (Table S7, supplemental material). We found that the two compounds were generally more active against *S. pseudintermedius* (2–16 µg/mL) than *S. aureus* (32–>64 µg/mL).

**Discussion**

The aims of this study were to (i) characterize the antimicrobial activity of the novel peptide-peptoid hybrid B1 against a large collection of two common dog integumentary pathogens *S. pseudintermedius* and *P. aeruginosa*; (ii) probe the mode of action against *S. pseudintermedius* E104; (iii) conduct a structure-activity study of B1 involving 28 analogues and identify compounds with potent activity against representative strains of *S. pseudintermedius* and *P. aeruginosa*, paired with weak activity against *S. aureus*; (iv) test the most promising of these analogues (23 and 26) as well as B1 against a broad panel of other canine pathogens; (v) test for selectivity within the *Staphylococcus* genus between *S. pseudintermedius* and *S. aureus*.

B1 showed low MICs against 50 canine *P. aeruginosa* (8–16 µg/mL), 57S. *pseudintermedius* (2–4 µg/mL). Killing kinetics showed that B1 kills MRSP and *P. aeruginosa* in less than 30 min at 8 x MIC. Our MIC and time-kill data for MRSP and *P. aeruginosa* are comparable with previous literature reports. Mohamed *et al.* designed and tested synthetic peptides (8 to 16 amino acids) against MSSP and MRSP. The most effective peptides displayed a MIC<sub>30</sub> and MIC<sub>90</sub> of 1 and 2 µM, respectively. Molchanova *et al.* reported 22 different α-peptide/β-peptoid hybrids containing cationic and hydrophobic residues in a 1:1 ratio that were active against MRSP (2–8 µg/mL) as well as other relevant Gram-positive and Gram-negative bacteria. The same authors also identified fluorinated antimicrobial lysine-based peptidomimetics with activity against methicillin-resistant *S. pseudintermedius*<sup>29</sup>. Finally, Cabassi and coworkers identified a peptide (AMP2041) with activity against human and animal multidrug resistant *P. aeruginosa* isolates<sup>31</sup>, including strains of canine origin. In a parallel study to the present, we have characterized the *in vitro* pharmacokinetic properties of B1, including hemolytic activity and stability to proteases. The measured hemolytic activity of B1 was 32% at 150 µM and the compound showed only 38% degradation after 24 hours exposure to the mix of protease of bacterial origin Pronase. Furthermore, B1 was suitable for topical delivery from cream formulation and showed no skin penetration after administration<sup>30</sup>. These data, combined with our results from the present study, suggest that B1 may be suitable as antimicrobial for topical treatment of canine superficial pyoderma.

To investigate the mode of action of B1, we used the well-characterized clinical MRSP ST71 strain E104 (MIC = 8–16 µg/mL), which is resistant to β-lactams, ciprofloxacin, clindamycin, doxycycline, and trimethoprim/sulfamethoxazole<sup>3</sup>. We found that the primary mode of action of B1 is on the bacterial membrane and secondarily on cell wall synthesis. However, B1 has no effect on DNA replication. Membrane activity was supported by a reduction of the initial OD in the growth curve study at 3 µg/mL (Fig. 5, Supplementary information), indicating cell lysis. Furthermore, B1 caused significant membrane depolarization as seen by an increased DiSC<sub>3</sub>(5) fluorescence emission (Fig. 5a,b). We obtained further support for the membrane permeabilization by the rapid cell death observed in the flow cytometry assay (Fig. 5).

The primary mode of action of B1 is in agreement with the classical membrane-targeting mechanism reported for a number of antimicrobial peptides, e.g. magainin II<sup>22</sup> and Cecropin B<sup>35</sup>. Besides membrane disruption, AMPs may have intracellular targets as reviewed recently<sup>4</sup>. These include protein<sup>33</sup>, DNA<sup>4</sup>, and cell-wall synthesis<sup>37</sup>. Here, B1 reduced cell wall synthesis by 20% (Fig. 3b). A few antimicrobial peptides such as plectasin have been reported to inhibit cell wall synthesis in Gram-positive bacteria. Plectasin targets the bacterial cell wall precursor lipid II as determined by advanced NMR<sup>38</sup>

Our finding that B1 does not affect DNA synthesis (Fig. 3a), is in agreement with literature reports that most AMPs do not have this target. However, some exceptions are known, e.g. indolicidin<sup>40</sup>. Furthermore, LPS, a compound similar to B1, has been reported to inhibit DNA replication and induce SOS response in *S. aureus*<sup>40</sup>. This may be due to a difference in net charge +4 (B1) and +6 (LPS), respectively.

In the mechanism of action study of B1, we used the well-characterized antimicrobial agent nisin as control. This antimicrobial compound used in food preservation interacts with the peptidoglycan precursor molecule lipid II, which leads to membrane depolarization and bacterial cell death<sup>41</sup>. The mechanism of nisin has been studied using a plethora of different techniques<sup>42</sup>. In our study, nisin had an inhibitory effect on MRSP growth at sub-lysis concentrations (Fig. S6 Supplementary Information). Simultaneous cell lysis and growth inhibition effects by nisin may be linked to the dual mode of action of this AMP, which encompasses both membrane pore formation and cell wall synthesis inhibition. Exposure to nisin resulted in 60% inhibition of cell wall synthesis, and 50% inhibition of DNA synthesis (Fig. 3a,b). We did not investigate the mechanism of B1 on *P. aeruginosa*, but a few previous reports on the AMP killing mode of action of *P. aeruginosa* exist: using fluorescence microscopy and field emission scanning electron microscopy, Memariani *et al.* found that the 14-mer AMP PV3, kills bacteria by disrupting the cell membrane<sup>41</sup>. Furthermore, Scocchi and coworkers investigated the mechanism of killing against *P. aeruginosa* strain PAO1 and additional three isolates and observed that Bac7 (1–35) inactivated the target cells by disrupting their cellular membranes<sup>41</sup>

We synthesized 28 analogues of B1 by (i) altering the peptoid residues position in the sequence; (ii) substituting peptoid residues for L-amino acids; (iii) modifying the chirality of the amino acid components and/or altering their position in the sequence. Like B1 and most AMPs, these analogues are cationic and hydrophobic. The rationale for their design is further discussed in results section. Besides *S. pseudintermedius* and *P. aeruginosa*, all the analogues were also tested against MSSA (ATCC 29212). Our two best compounds, 23 and 26, showed slightly higher MICs (Table 1) against *S. pseudintermedius* (2–4 µg/mL and 4–8 µg/mL respectively) and *P. aeruginosa* (8 µg/mL) compared to B1 and considerably worse activity against MSSA (32–64 µg/mL, >64 µg/mL), respectively.
Hemolysis at 150µM is a commonly used parameter in the literature for comparison of antimicrobial peptides or peptidomimetics. The hemolytic activity of 23 and 26 was 59% and 56% at 150µM, respectively. A variation of the therapeutic index, selectivity index, SI, is often used in the field and is defined as the ratio between the concentration leading to 50% lysis of human erythrocytes and the minimum concentration inhibiting bacterial growth SI = (HC50/MIC) for the bacterium being considered. The selectivity indices for MRSP C22963 are: B1 (82), 23 (37), 26 (11). Typically, selectivity indices are below 100, although higher values have been reported.

The SI values are not a major issue for drugs intended for topical use, since a highly hemolytic compound like the steroid antibiotic fusidic acid is being used for against human skin disorders and canine pathogenic staphylococci. Compound 23 showed slower killing kinetics against MRSP C22963 and P. aeruginosa than B1, and was not able to eliminate P. aeruginosa but resulted in delayed re-growth. Notably, compound 26 was able to kill MRSP C22963 within 1h at only 0.5x MIC, which was faster than both B1 and 23 and fully comparable with literature reports.

We tested the activity of B1 and the analogues 23 and 26 against a broader range of canine pathogens. They displayed antimicrobial activity (Table 2) against a collection of Gram-positive clinical isolates (2- >64µg/mL) and less against the Gram-negative isolates (8- >64µg/mL). Generally, B1 proved a lot more active than 23 and especially 26 (Table 2). In addition to S. pseudintermedius, S. aureus and P. aeruginosa, we observed increased activity for B1 (4–16µg/mL) against other bacteria such as: Corynebacterium auriscanis associated with canine otitis externa, Streptococcus canis which causes respiratory, cutaneous, genital and urinary infections in various animal species, Pasteurella canis which is a well-known major pathogen of infections caused by dog bites, and Enterococcus faecium which is an important nosocomial pathogen. However, we observed no significant activity against bacterial isolates belonging to the species Acinetobacter baumannii, Escherichia coli, Enterococcus faecalis and Klebsiella pneumoniae.

Having established the antimicrobial spectrum of 23 and 26, we tested the selectivity of 23 and 26 against clinical isolates of S. pseudintermedius (n = 10) vs S. aureus (n = 10) (Table S7, supplementary information). We found that the two compounds were generally more active against S. pseudintermedius (2- >16µg/mL) than S. aureus (2- >64µg/mL), especially compound 26. AMPs with enhanced selectivity against a target bacterial genome have been previously reported and selectivity within a genus has been observed in few other studies. For example, Guo et al. published a peptide, C16G2, which is able of killing S. mutans selectively but not closely related streptococcal species. Our finding is significant, as we demonstrated that short amphiathic peptidomimetics can maintain activity against S. pseudintermedius, even when their efficacy against S. aureus decreases multiple folds. This suggests that the former might be more susceptible to membrane-active agents, or susceptible to a wider range of agents. These observations should aid the design of novel therapeutics for the treatment of S. pseudintermedius infections in animals, for which B1, 23 and 26 pose as promising lead candidates. The underlying reason for the higher activity of compounds 23 and 26 against S. pseudintermedius over S. aureus is unknown, but might be related to peptides mechanism of interactions with the bacterial cell envelope. The spatial orientation of amino acid side-chains upon interacting with the bacterial membrane is often fundamental for the activity of AMPs. The compounds B1, 23 and 26 all contain seven residues, which is too short to display α-helical structure. Therefore, they most likely form random coils. Furthermore, 26 contains D-amino acids, which are known to disrupt α-helix structure. Finally, the presence of even a single peptoid residue in an α-helix has been reported to result in a significant reduction of the helical content. Similarities and differences among bacterial membranes of different species are crucial in determining the spectrum of activity of amphipathic AMPs. Since the structure of B1, 23 and 26 are closely related, we speculate that the mechanism of action of 23 and 26 is similar to that of B1. Compound B1 is an L-peptide/peptoid hybrid, 23 is a full L-peptide containing non-canonical amino acids, whilst 26 is an L/D-peptide hybrid (Fig. 1). The affinity of antimicrobial peptides for bacterial cells is due to their amphiphilic properties (hydrophobic and positively charged). The interaction with the bacterial membrane is regulated, for both peptides and peptoids, by their affinity of antimicrobial peptides for bacterial cells is due to their amphiphilic properties (hydrophobic and positively charged). The interaction with the bacterial membrane is regulated, for both peptides and peptoids, by their interaction with the bacterial membrane is often fundamental for the activity of AMPs.

Veterinary medicine needs antimicrobials which are tuned to a veterinary spectrum and are not shared with human medicine. These considerations are in line with the “One Health” view of infectious diseases, which acknowledges that humans and animals share the same pool of bacterial pathogens. Moreover, MRSP has been isolated in humans, highlighting its zoonotic potential and therapeutic challenge. Therefore, it is in the interest of veterinary medicine and public health that novel antimicrobial agents target P. aeruginosa and S. pseudintermedius in companion animals selectively and not closely related species within a genus, including S. aureus.

In this perspective, we have characterized a novel peptide-peptoid hybrid B1 with antimicrobial activity against both S. pseudintermedius and P. aeruginosa, the main cause of pyoderma in dogs. A structure-activity study identified two compounds, 23 and 26, with potent activity against the aforementioned species, paired with poor activity against the closely related S. aureus. Our results represent a first step towards the design of peptides and peptidomimetics with a pathogen-targeted spectrum, even within a genus. Such investigation deserves further exploration towards the rationale design of drugs selective for veterinary pathogens.

Materials and Methods

Synthesis of peptides and peptidomimetics. The synthesis of the peptides and peptidomimetics was performed as described by Oddo et al. Briefly, peptides were synthesized by Fmoc solid phase peptide synthesis (SPPS). Peptidomimetics were prepared by a combination of the above and sub-monomer peptoid synthesis. Following TFA-cleavage, precipitation in ether and lyophilisation, the compounds were purified (>95%) by preparative HPLC and the purity was determined through analytical HPLC. The identity of each compound
was verified by MALDI-TOF-MS. The compounds used in this study are shown in Supplementary Information (Table S1).

Antimicrobial susceptibility testing. Activity of B1 was tested on 50 P. aeruginosa isolates and 57 S. pseudintermedius isolates (including 7 MRSP) that had been isolated in the diagnostic laboratory Sund Vet Diagnostik (University of Copenhagen) from various infections in dogs between 2009 and 2011. Representative MRSP (C22963), MSSA, and P. aeruginosa (26314) were used for testing the first set of B1 analogues.

For antibacterial spectrum the following strains were used: Corynebacterium aurisicanis, 31551, (54 C6, dog, ear, 2013); Enterococcus faecalis, 27404, (17 C7, dog, wound, 2011); Enterococcus faecium, 30951, (24 C1, dog, ear, 2013); Streptococcus canis, 26740-1, (14 H1, dog, ear, 2010); Staphylococcus aureus, 27266, (16 G9, dog, skin, 2010); Staphylococcus aureus, 27266, (16 G9, dog, skin, 2010); Staphylococcus pseudintemedius, 27266, (16 G9, dog, skin, 2010); Staphylococcus pseudintermedius, 22963, (3 B9, dog, 2007). Acinetobacter baumannii, 27065, (16 D1, dog, wound, 2010); E. coli, 30235, 23 A6, dog, wound, 2012; Klebsiella pneumoniae, 26233, (11 H5, dog, wound, 2010); Pasteurella canis, 31096, (24 C8, dog, skin, 2013); Proteus mirabilis, 25178, (9 A4, dog, ear, 2009); Pseudomonas aeruginosa 26314, 12 C5, dog, urine, 2010.

For testing the selectivity of 23 and 26 against clinical isolates of S. pseudintermedius vs S. aureus the following strains were used.

S. aureus strains. 25054, (8 G6, dog, wound, 2009); 27266, (16 G9, dog, skin, 2010 also used in exp above); 28264,(20 B1, dog, wound, 2011); 30935, (24 B9, dog, joint, 2013); 36968, (61 A9, dog, wound, 2016); 37595,(65 D2, dog, joint, 2016); 37708-2, (66 C6, dog, skin, 2016); 38200, (68 E9, dog, skin, 2016); 38565-1, (70 A5, dog, skin, 2017); 38841, (70 G5, dog, urine, 2017).

S. pseudintermedius strains. 26071, (11 E5, dog, skin, 2009); 26092-2, (11 E4, dog, skin, 2009); 26959, (15 F8, dog, wound, 2010); 27364, (17 A7, dog, wound, 2011); 27382, (17 B8, dog, ear, 2011); 27382, (17 B8, dog, ear, 2011); 31524, (54 C3, dog, ear, 2013); 33228, (55 E2, dog, skin, 2014); 35890, (59 B6, dog, ear, 2015); 37526-1, (65 B4, dog, skin, 2016); 37535-1, (65 B6, dog, skin, 2016); 37535-1, (65 B6, dog, skin, 2016); 38637, (70 C3, dog, wound 2017); 38820, (70 F9, dog, urine 2017).

MIC determination was performed by broth microdilution according to the Clinical and Laboratory Standard Institute (CLSI, M31-A3, 2008)64. In brief, each bacterial strain was diluted to concentration of 1 to 64 μg/ml in 96-well plates (Nunc Internationals, Rochester, NY). The MICs were determined as a lowest concentration showing no visible growth after incubation for 18 hours at 37°C. Experiments were performed in triplicates on two different days.

Time kill curves. Time kill assays were performed in triplicate, meaning that every value is the average of three different samplings. Time–kill kinetic assays were performed using P. aeruginosa (26314), MRSP E104 and/or MRSP C22963 to determine the cell killing activity of B1 and two of its most promising analogues (compounds 23 and 26) based on prior antimicrobial susceptibility testing. The method reported by Blondeau et al.65 was followed with minor modifications. Briefly, the assay was performed in MHB II with concentrations corresponding to 0.5, 1, 2 and 4 times the MIC of the strain. After 0, 15, 30, 60, 120, 180 and 300 min of growth, 100 μL aliquots of cell suspension from each dilution were spotted in triplicate on blood agar plates followed by 16–18 h incubation at 37°C and determination of colony forming units (CFU).

Haemolytic activity. The EC10 (10% maximal effective concentration), EC50 (half maximal effective concentration) values and the percentage of haemolysis at 150 μM were determined for all compounds as previously described64. Briefly, two-fold serial dilutions (2.35 to 150 μM) of compounds in phosphate buffer saline (PBS) were mixed with equal volume of 0.5% v/v suspension of fresh human red blood cells (RBC) in the same buffer. After 1 h incubation at 37°C, plates were centrifuged and aliquots of the supernatants were transferred to clear 96-well plates. Absorbance at 414 nm was measured and normalized using a negative (PBS, 0%) and a positive (melittin, 100%) control. The EC10 and EC50 are concentrations at which 10% and 50% of RBC were lysed, respectively, as interpolated graphically by the y-axis intersection of the plotted data.

Macromolecule biosynthesis rate. Macromolecule biosynthesis rate was measured in MRSP E104 following a protocol adapted from Ling et al.65. Briefly, E104 overnight culture was sub-cultured 1:100 in MHB II and grown up to OD 0.2 at 600 nm. Cells were pelleted down by centrifugation and resuspended in fresh medium followed by incubation for 20 min with B1 or nisin at 1.5 μg/ml and 1.25 μg/ml, respectively, and radiolabeled precursor: (5μCi) 3H-Thymidine (PerkinElmer) and (5μCi) 3H-glucosamine hydrochloride per ml for DNA and cell wall, respectively. A positive control without antimicrobial was maintained. After incubation, samples were precipitated with equal volume of cold 30% TCA (Sigma) on ice. Precipitates were filtered on a membrane filter and subsequently subjected to two washes of cold 15% TCA and two washes of cold water using vacuum manifold. Subsequently, filters were air dried overnight and then transferred to 10 ml scintillation vials. Finally, scintillation fluid (3 ml) was added to each vial and 3H count was taken in Beckman Coulter LS6500 liquid scintillation counter for one minute. The radioactive counts of the control samples were considered to have 100% precursor incorporation and macromolecule synthesis. The percentage rates of the antimicrobial-exposed samples were calculated accordingly. Each experiment was performed with replicates and the average rates of incorporation were plotted.
DiSC3(5) fluorescence-based membrane potential study. Freshly sub-cultured E104 cells were labelled with 1 µM 3,3'-Dipropylthiadicarbocyanine iodide [DiSC3(5)] (Sigma) in MHB II. The fluorescence spectra of labelled cells were plotted in the LS50B luminescence spectrometer (PerkinElmer) at excitation/emission wavelengths 546 nm/573 nm using time drive application of FLWinLAB software. After reading an initial stable emission spectra of DiSC3(5), labelled cells were treated with B1 or nisin or protonophore CCCP at 1, 1.5 and 1.25 µg/ml, respectively and the change of fluorescence over time was recorded. The increment of DiSC3(5) fluorescence upon addition of antimicrobials or CCCP (FUafter-treatment – FUbefore-treatment) was plotted. This experiment was performed twice and each time with two technical replicates.

Flow cytometry analysis of antimicrobial-exposed cells. Freshly grown cultures (OD 0.2 at 600 nm) of MRSP E104 were exposed to 3 µg/ml of B1 or 2.5 µg/ml of nisin for 1 h and diluted 1:10 in flow cytometry analysis buffer (PBS supplemented with 1 mM EDTA, 0.01% Tween 20 and filtered by 0.22 µm membrane). Diluted cells were stained with 420 nM thiazole orange (TO, Sigma) and 48 µM propidium iodide (PI, Sigma) and analysed using BD ACCURI C6 flow cytometer (BD Biosciences). Unstained cells were used as negative control. Finally, the differentially labelled bacterial populations in antimicrobial-exposed cultures were plotted under FL3(red)/FL1(green) axis using the instrument’s software and categorised into four different conditions: P1 (unstained), P2 (dead cells), P3 (injured cells) and P4 (live cells).

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**Author Contributions**

I.G., A.E., B.J. N.M. and P.D. performed the experiments. I.G., A.E., B.J. N.M. A.O., P.D., L.G. and PRH conceived and designed the study. I.G., B.J. N.M., P.D. and P.R.H. wrote the manuscript. All authors contributed to the final version of the manuscript and approved it.

**Additional Information**

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