ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2007, p. 1837–1839 Vol. 51, No. 5
0066-4804/07/$08.00+0 doi:10.1128/AAC.00924-06

Efficacy of the De Novo-Derived Antimicrobial Peptide WLBU2 against Oral Bacteria▽

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Received 26 July 2006/Returned for modification 6 October 2006/Accepted 12 February 2007

The efficacy of a novel synthetic antimicrobial peptide (WLBU2) was evaluated against three oral microorganisms (grown planktonically): *Streptococcus gordonii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. WLBU2 killed all three species, with *F. nucleatum* being the most susceptible. WLBU2 also reduced the bacterial burden of *S. gordonii* and *F. nucleatum* biofilms.

With increasing evidence for links between infectious burden and systemic diseases such as coronary artery disease (6, 15, 18), the oral cavity has been shown to be an important contributor to this systemic microbial bioburden (4, 5, 7, 8, 12). The microcosm found in the oral cavity comprises more than 700 species of microorganisms (1) arranged as both single-species and multispecies biofilms. The tooth-associated biofilm (dental plaque) is the primary etiologic factor associated with dental caries and periodontal disease. Dental plaque biofilm formation is a sequential process initiated by adherence of gram-positive early colonizers to the tooth surface, followed by a shift to a predominance of more pathogenic gram-negative anaerobic species (late colonizers) as the biofilm matures. Importantly, *Fusobacterium nucleatum* serves as an important coaggregation bridge between early and late colonizers (9).

The use of topical chemotherapeutic agents that inhibit plaque formation is an important adjunct in the prevention of oral diseases such as dental caries and periodontal disease. Therefore, the development of novel antimicrobial compounds that are effective against oral microorganisms is an important aspect of both basic science and commercial oral health research. The lentivirus lytic peptides are one example of this class of novel compounds (11, 13, 17). A de novo-engineered derivative of lentivirus lytic peptide, WLBU2, is a 24-amino-acid peptide that is effective against a broad spectrum of gram-positive and gram-negative isolates (2). WLBU2 eliminates *Pseudomonas aeruginosa* in coculture with human skin fibroblasts without detectable adverse effects to the host cells, is active against *Staphylococcus aureus* and *P. aeruginosa* at physiologic NaCl concentrations, and kills *P. aeruginosa* in human serum. WLBU2 also eradicates bacteria from ex vivo samples of whole blood, suggesting it may be suitable for use as a topical or systemic chemotherapeutic agent in the prevention and treatment of medically important infections (3). Based on the need for the development of chemotherapeutics against oral pathogens and the demonstrated antimicrobial activity of WLBU2, we hypothesized that oral bacteria would be sensitive to this peptide.

Using a standard broth dilution assay (17), the potency of WLBU2 was evaluated against planktonic cultures of *Streptococcus gordonii*, an early colonizer of dental plaque; *F. nucleatum*, the important coaggregation "bridging" colonizer; and *Porphyromonas gingivalis*, a late colonizer associated with multiple forms of periodontal disease (9, 14). *P. aeruginosa* was used as a positive bacterial control. Bacterial cultures were propagated in liquid media under appropriate growth conditions (Table 1) to mid-log phase, washed with phosphate buffer (PB), and suspended in PB such that upon dilution, 10^5 to 10^6 CFU/ml was tested in the bacterial killing assay. The bacteria were incubated with twofold dilutions of the peptide (100 μM to 0.39 μM) in 96-well plates in PB at 37°C under appropriate growth conditions (Table 1). Although WLBU2 reduces viable counts of *P. aeruginosa* in seconds in PB (17), a minimum of 20 min is required for killing in serum (3). Since the subgingival environment contains a serum transudate, a standard 30-min peptide exposure was selected for this pilot project. Quantification of bacterial survival post-peptide exposure was evaluated using serial 10-fold dilutions of control and test wells. Bacterial colonies were counted at 24 h for *S. gordonii* and *P. aeruginosa* and at 48 h for *F. nucleatum* and *P. gingivalis* and were compared to counts of non-peptide-treated controls to determine the amount of WLBU2 that reduced the bacterial counts by 3 orders of magnitude. This level of killing defined the minimum bactericidal concentration (MBC), assessed in micromolar concentrations of peptide. The results were expressed as averages of MBCs obtained from three independent experiments.

All three species of planktonic oral bacteria were killed by WLBU2 (Fig. 1). The MBC was between 1 and 2 μM for *P. aeruginosa* and *F. nucleatum*, between 12.5 and 25 μM for *S. gordonii*, and between 50 and 100 μM for *P. gingivalis*. Viable counts also were assessed for *S. gordonii* and *F. nucleatum* following exposure to comparable micromolar concentrations of amoxicillin or metronidazole, antibiotics frequently used in treating periodontal disease (16). There were no significant

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▽ Published ahead of print on 26 February 2007.

TABLE 1. Bacterial strains and growth conditions

Bacterial strain	Medium	Growth conditions
Pseudomonas aeruginosa ATCC 47085	Todd-Hewitt broth	Aerobic
Streptococcus gordonii ATCC 49818/DL1	Todd-Hewitt broth	Aerobic
Fusobacterium nucleatum ATCC 49256, ATCC 25586	Tryptic soy broth + 0.6% yeast extract	Anaerobic (5% CO_2, 10% H_2, 85% N_2)
Porphyromonas gingivalis ATCC BAA0308/W83	Mycoplasma broth + hemin (5 μg/ml) + menadione (1 μg/ml)	Anaerobic (5% CO_2, 10% H_2, 85% N_2)

decreases in the viable cell counts of either bacterium following these treatments (Tables 2 and 3).

The high concentration required for killing *P. gingivalis* strain W83 may be due to the encapsulated nature of this strain or the presence of the numerous proteolytic enzymes released by this bacterium. This will be assessed in future studies. However, the MBCs for two different strains of *F. nucleatum* were between 1 and 2 μM, consistent with results evaluating the specificity of WLBU2 against numerous *P. aeruginosa* strains (2). Interestingly, both strains of *F. nucleatum* were far more susceptible to killing by this novel peptide than either *S. gordonii* or *P. gingivalis* when grown as planktonic cells. Because *F. nucleatum* is recognized as an important "bridging" bacterium between the less pathogenic early colonizers and the more pathogenic late colonizers of the plaque biofilm, antimicrobial agents disrupting this bridge before the development of a mature biofilm could interfere with plaque maturation.

The effectiveness of WLBU2 was assessed against single-species oral biofilms developed on individual rigid gas-permeable lens (RGPL) material (unpublished data). Replicate RGPLs were coated with fetal bovine serum, washed with sterile phosphate-buffered saline (PBS), and incubated with overnight cultures of *S. gordonii* or *F. nucleatum* for 24 h under appropriate conditions (Table 1). After 24 h, the culture medium was carefully removed without displacing the developing biofilm and was replaced with an equal volume of fresh medium daily for 3 days (*S. gordonii*) or 4 days (*F. nucleatum*). The RGPL material was then washed with sterile PBS to remove nonadherent cells and treated with either WLBU2, amoxicillin, or metronidazole (25 μM for *S. gordonii*; 2 μM for *F. nucleatum*) for 30 min under appropriate conditions (Table 1). Additional replicates treated with PBS served as controls. Treated and control biofilms were released from the RGPL into PBS with a sterile cell scraper and disrupted by vortexing. Serial dilutions were spread onto blood agar plates in triplicate for determination of CFU.

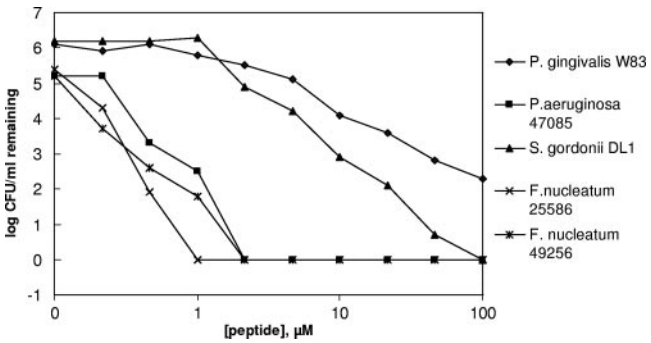

FIG. 1. Dose-dependent killing of oral bacteria and *P. aeruginosa* (positive control) by WLBU2. Bacterial cultures (0.5×10^6 to 1×10^6 CFU/ml) were treated with twofold dilutions of the peptide in phosphate buffer; the log of the number of CFU/ml remaining upon treatment is plotted as a function of peptide concentration.

TABLE 2. Viable counts of untreated (PBS) versus treated *S. gordonii* cells

Method of cell growth	Treatment[a]	Total viable count	P^b
Planktonic	PBS	$(1.29 \pm 0.25) \times 10^4$	
	WLBU2	$(0.00 \pm 0.00) \times 10^4$	<0.001
	Amoxicillin	$(1.35 \pm 0.41) \times 10^4$	NS
	Metronidazole	$(1.22 \pm 0.11) \times 10^4$	NS
Biofilm	PBS	$(2.01 \pm 0.08) \times 10^7$	
	WLBU2	$(0.21 \pm 0.02) \times 10^7$	<0.0001
	Amoxicillin	$(1.63 \pm 0.11) \times 10^7$	NS
	Metronidazole	$(1.8 \pm 0.14) \times 10^7$	NS

[a] All antimicrobials were used at a concentration of 25 μM.
[b] NS, not significant; $P > 0.05$ by a *t* test that was adjusted when necessary for unequal variance.

The results of three replicate experiments demonstrated that treatment of *S. gordonii* biofilms with WLBU2 resulted in a statistically significant decrease in mean cell counts compared to those for PBS-treated controls. Mean cell counts did not decrease significantly following treatment with either amoxicillin or metronidazole (Table 2). Similarly, treatment of *F. nucleatum* biofilms with these antibiotics did not decrease viable cell counts, while treatment with WLBU2 did lead to decreased counts. However, the results with WLBU2 were not statistically significant (Table 3). Taken together, these results indicate a trend toward the ability of WLBU2 to impact viable counts of both *S. gordonii* and *F. nucleatum* cells grown as biofilms.

These studies demonstrate activity of WLBU2 against early and bridging bacteria grown as planktonic cells and known to be part of oral biofilms. Our results also demonstrate that WLBU2 can potentially contribute to a lessening of the bioburden of a mature single-species biofilm. We hypothesize that the somewhat limited effect of the peptide on biofilm cells was due to inefficient penetration beyond the surface layer of bacteria in the mature biofilm. Consistent with our findings and this hypothesis, it has been demonstrated previously that mechanical disruption or adjunctive exposure to a surface-active agent

TABLE 3. Viable counts of untreated (PBS) versus treated *F. nucleatum* cells

Method of cell growth	Treatment[a]	Total viable count	P^b
Planktonic	PBS	$(1.04 \pm 0.08) \times 10^7$	
	WLBU2	$(0.00 \pm 0.00) \times 10^7$	<0.0001
	Amoxicillin	$(1.00 \pm 0.15) \times 10^7$	NS
	Metronidazole	$(1.02 \pm 0.07) \times 10^7$	NS
Biofilm	PBS	$(1.75 \pm 0.13) \times 10^4$	
	WLBU2	$(0.20 \pm 0.27) \times 10^4$	NS
	Amoxicillin	$(2.75 \pm 0.07) \times 10^4$	NS
	Metronidazole	$(2.86 \pm 0.57) \times 10^4$	NS

[a] All antimicrobials were used at a concentration of 2 μM.
[b] NS, not significant; $P > 0.05$ by a *t* test that was adjusted when necessary for unequal variance.

can enhance peptide killing of biofilm cells (10). Therefore, future studies will evaluate the efficacy of WLBU2 in combined therapeutic strategies with a focus on the peptide's impact on *F. nucleatum* as a coaggregating bridge microorganism in multispecies biofilms.

This work was supported by a University of Kentucky research support grant and by Kentucky Science and Technology Corporation/Kentucky Science and Engineering Foundation grant KSEF-47-RDE-004.

We thank Jeff Mattingly for valuable technical assistance with this project, Malini Bharadwaj for assistance with manuscript preparation, and Kazi Islam at the University of Pittsburgh Molecular Medicine Institute Peptide Synthesis Facility for assistance in peptide preparation.

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