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ANTIBACTERIAL EFFECTS AND CYTOTOXICITY OF TWO DERMASEPTIN DERIVATIVES

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DECLARATION OF INTEREST

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ABBREVIATIONS

µg	Microgram
µl	Microliter
AMP	Antimicrobial peptide
BM2	Basal media two
CFU	Colony forming units
HGEP	Human gingival epithelium progenitors
M9	M9 salts minimal media
MDR	Multidrug resistant
MH	Mueller-Hinton
MIC_{90}	Minimum inhibitory concentration, 90%
ml	Milliliter
mM	Millimolar
OD_{600}	Optical density to 600 nanometer wavelength light
TSB	Tryptic soy broth
WST-1	Water soluble tetrazolium salt one

EXECUTIVE SUMMARY

Background: Food and Drug Administration approvals of novel antibiotics are uncommon, especially for antibiotics against Gram-negative bacteria, and rates of nosocomial, multidrug resistant infections are increasing. This generates a gap between our need for antibiotics and the number of effective antibiotics available. One way to address this widening gap is to search for antimicrobial peptides, small proteins with broad spectrum antibacterial effects.

Objective: Test two antimicrobial peptides: the dermaseptin derivatives $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$, for efficacy against clinical isolates and laboratory strains of the Gram-negative pathogen *Pseudomonas aeruginosa* grown in various growth media and for cytotoxicity in human gingival epithelium progenitor cells.

Methods: The two antimicrobial peptides were tested against four *P. aeruginosa* strains: the common laboratory strain PAO1, a more virulent laboratory strain PA14, and multidrug resistant clinical isolates 105734 and 105765, grown in four different media compositions: the minimal media basal media two, the minimal media M9 salts media, the complete media tryptic soy broth, and the complete media Mueller-Hinton broth.

Results: Both antimicrobial peptides are more effective against *P. aeruginosa* grown in minimal media than in complete media. Overall, $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$ are just as effective against multidrug resistant clinical isolates as they are against laboratory strains.

Conclusions: Both $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$ can be effective against multidrug resistant isolates and laboratory strains of *P. aeruginosa*. The shorter peptide, $K_4S_{4(1\text{-}15)}$, is both less cytotoxic and more efficacious than $K_4K_{20}S_4$, making it an especially good candidate for future antibacterial development.

INTRODUCTION

Chronic wound infections involving multidrug resistant (MDR) pathogens, especially nosocomial infections from military/civilian hospitals or clinical settings, have become commonplace nationwide. To combat these MDR infections, hospitals are forced to implement infection control practices, controlled antibiotic use, and active culture surveillance techniques due to a dearth of new antibiotics (Boucher HW 2009). However, developing new drugs involves large expenditures of time, research effort, and expense. Only a small number of patients who contract MDR infections meet the requirements to participate in clinical trials for new drugs (Pew Charitable Trusts 2014), and 80% of drugs that begin initial human testing are not approved by the Food and Drug Administration (Hay et al. 2014). Thus, the challenges of developing new antibiotics for highly resistant bacterial infections have narrowed the drug pipeline to only a few new antibiotics per year (Spellberg B 2008). The combination of these difficulties and the growing number of MDR infections prompted research for alternative antimicrobial products.

One approach is to search for antimicrobial peptides (AMPs), small proteins with broad-spectrum antibacterial effects (de la Fuente-Nunez et al. 2014) and multidirectional modes of action that make them less likely to induce bacterial resistance (Chan, Prenner, and Vogel 2006). Hundreds of AMPs are reported to provide potent, broad-spectrum activity against microbial infections (Segev-Zarko et al. 2015; de la Fuente-Nunez et al. 2014; Dixon DR 2008), and some of these are the subject of significant attention as potential therapeutics for antibiotic resistant infections. Many organisms use AMPs to combat infectious parasites, viruses, and fungi (Organization April 2015). The Database of Antimicrobial Activity and Structure of Peptides v2.13 (Pirtskhalava et al. 2016) lists more than 11,500 AMPs (dbaasp.org/home, accessed 2 Jan

2019). These AMPs originate from animals, plants, fungi, and bacteria (Andreu D 1998; Reddy KV 2004; Guani-Guerra E 2010), but many share common characteristics. For example, most positively charged AMPs interact selectively with the anionic bacterial cell membranes while having much less affinity for the zwitterionic eukaryotic cell membrane (Malmsten 2014; Schmidtchen et al. 2009). While the exact mechanisms of action of these AMPs are not fully understood, they are known to act generally by altering the membrane potential (Han et al. 2013), inducing cell leakage through transmembrane holes (Gee et al. 2013), disrupting native membrane lipid distribution (Matsuzaki et al. 1996), and inducing autolysis in bacteria (Bierbaum and Sahl 1985). Whatever the mechanism, AMPs cause membrane function breakdown followed by bacterial death (Wimley 2010).

One of the most intensively studied groups of AMPs are the dermaseptins, a superfamily of host defense peptides made in the skin of Hylidae frogs (Nicolas and Ladram 2013). Most AMPs in this group have broad spectrum antibacterial activities at micromolar concentrations. Derivatives of dermaseptin S_4 are known for especially high antibacterial activity against *P. aeruginosa*, as well as *Streptococcus mutans* and *Candida albicans*, two oral pathogens (Porat et al. 2006), making them attractive for potential use in the oral cavity or against MDR infections. Their mode of action results from the permeation/disruption of the bacterial plasma membrane (Ladram and Nicolas 2016). We tested two dermaseptin S_4 derivatives, $K_4K_{20}S_4$ (Zairi et al. 2014) and $K_4S_{4(1\text{-}15)}$ (Porat et al. 2006) for efficacy against four strains of the opportunistic pathogen *Pseudomonas aeruginosa*. These included the common laboratory strain PAO1 (Stover et al. 2000), a more virulent laboratory strain PA14 (Mikkelsen, McMullan, and Filloux 2011), and strains 105734 and 105765 from the military health system, which are extremely tolerant of most commercially available antibiotics including ampicillin, aztreonam, cefazolin, cefepime,

cefoxitin, ceftriaxone, cefuroxime, nitrofurantoin, piperacillin, tetracycline, rifampicin, vancomycin, clindamycin, clindamycin, cephalexin, minocycline, and linezolid (unpublished data).

We tested each AMP's ability to inhibit *P. aeruginosa* growth in four different media: basal media two (BM2), M9 salts minimal media (M9), tryptic soy broth (TSB), and Mueller-Hinton (MH) media. Both BM2 and M9 are types of minimal media, while TSB and MH are both complete media. We also evaluated each AMP for cytotoxic effects in human gingival epithelium progenitor cells (HGEPs).

MATERIALS AND METHODS

Materials

Antimicrobial peptides. Dermaseptin derivatives $K_4K_{20}S_4$ (ALWKTLLKKVLKAAAKAALKAVLVGANA-NH_2) (Zairi et al. 2014) and $K_4S_{4(1\text{-}15)}$ (ALWKTLLKKVLKAAA-NH_2) (Porat et al. 2006) were chosen for their small sizes and potentially high efficacies. After 9-fluronylmethoxycarbonyl/tert-butyl solid-phase peptide synthesis (with amidated C-terminals, Genemed Synthesis Inc., San Antonio, TX) the AMPs were stored at -80 °C until use.

Pseudomonas aeruginosa strains and media. Clinical isolates 105734 and 105765 were provided by the Brooke Army Medical Center Microbiology Research Laboratory. Laboratory strains PAO1 (Stover et al. 2000) and PA14 (Mikkelsen, McMullan, and Filloux 2011) were purchased from the American Type Culture Collection (Manassas, VA). All *P. aeruginosa* strains were grown at 37 °C with shaking at 250 rpm in BM2, M9, TSB, or MH (Becton Dickinson, Franklin Lakes, NJ) as previously described (Lemon et al. 2018).

Procedures

Antibacterial testing and calculations. Minimum inhibitory concentrations, 90% (MIC_{90}), were calculated as described previously (Andresen, Tenson, and Hauryliuk 2016; Knappe et al. 2010). Briefly, overnight cultures were diluted to 10^5 to 10^6 colony forming units (CFUs) per milliliter in the appropriate media supplemented with either AMP, and incubated in 96-well, flat-bottom, non-treated plates (Thomas Scientific, Swedesboro, NJ) at 37 °C in 5% CO_2. After 18 hours, each well's OD_{600}, less background, was recorded. These values were divided by OD_{600} values for control wells without AMP to calculate relative growth. Relative growth values were converted into percent survival and pooled to calculate mean percent survival and standard deviation for each concentration of AMP. Any group of experiments that did not inhibit at least 90% of bacterial growth at AMP concentrations of 256 µg/ml were recorded as MIC_{90} = 512 µg/ml.

Statistical analysis. Before antibacterial data were analyzed, we pooled all MIC_{90} values for both AMPs and used Prism 6's (GraphPad, San Diego, CA) iterative Grubbs's test (with alpha = 0.05) to identify outliers. This test found nine outliers: five MIC_{90} = 512 µg/ml, one MIC_{90} = 128 µg/ml, and three MIC_{90} = 64 µg/ml. We excluded these data from our analysis. Two-sample t-tests (assuming unequal variances) were used to determine if differences were significant ($p < 0.05$).

Cytotoxicity testing. As described previously (Lemon et al. 2018), cytotoxicity assays were conducted with the water soluble tetrazolium salt one cell proliferation reagent (WST-1, Roche Diagnostics GmbH, Mannhein, Germany). Briefly, HGEP (ZenBio Inc., Research Triangle Park, NC) were grown in CellnTec-Prime media (CellnTec, Bern, Switzerland) at 37 °C

and 5% CO_2. Initially, 1.25×10^4 cells were seeded into each well of a 96-well plate. After 24 hours, the cells were rinsed with PBS and fresh media supplemented with the appropriate concentration of either $K_4K_{20}S_4$ or $K_4S_{4(1\text{-}15)}$ was added. After 24 hours, WST-1 was used to determine HGEP survival. Triton X-100 was used to induce cell death as a positive control. Cells were incubated with WST-1 for two hours before survival was inferred from the ratio between OD_{440} and OD_{600}. Differences between these OD readings were pooled for calculating mean percent cell survival and standard deviation.

RESULTS

Effects of culture media on the antimicrobial activities of dermaseptin derivatives with *P. aeruginosa*. First, we tested each AMP for efficacy against *P. aeruginosa* laboratory strains PAO1 and PA14, and clinical isolates 105734 and 105765 in planktonic culture. Both $K_4K_{20}S_4$ (Figure 1) and $K_4S_{4(1\text{-}15)}$ (Figure 2) were proportionally more effective at inhibiting bacterial growth as the concentration of AMP in the growth media increased. The lowest concentration of AMP that inhibited 90% of bacterial growth (minimum inhibitory concentrations 90%, MIC_{90}, Figure 3), calculated from the dose-response data shown in Figures 1-2 revealed both $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$ were significantly more effective ($p = 2.645 \times 10^{-15}$ and $p = 5.063 \times 10^{-5}$, respectively) against cultures grown in minimal media (mean MIC_{90} 4.66 ± 4.80 and 4.03 ± 6.53 μg/ml, respectively) than in complete media (mean MIC_{90} 13.82 ± 7.86 and 7.73 ± 6.65 μg/ml, respectively).

Effects of dermaseptin derivatives on drug-susceptible and MDR clinical strains of *P. aeruginosa.* Peptide $K_4S_{4(1\text{-}15)}$ and $K_4K_{20}S_4$ were equally effective ($p > 0.05$) against laboratory (mean MIC_{90} 5.18 ± 6.34 μg/ml and 7.68 ± 6.99 μg/ml, respectively) and clinical isolates (mean MIC_{90} 5.18 ± 7.16 μg/ml and 8.89 ± 8.29 μg/ml, respectively, Figure 3). Both

peptides were generally more effective ($p < 0.05$) against clinical isolate 105765 than any other *P. aeruginosa* strain, though this was not the case when $K_4K_{20}S_4$ was used against cultures grown in M9 or TSB, or when $K_4S_{4(1\text{-}15)}$ was used against cultures grown in BM2 (Figure 3).

Antimicrobial activities and dose-dependent effects of $K_4S_{4(1\text{-}15)}$ and $K_4K_{20}S_4$ dermaseptin derivatives. Comparing the two peptides to one another, $K_4S_{4(1\text{-}15)}$ performed better than $K_4K_{20}S_4$ against clinical isolates (mean MIC_{90} 5.99 ± 7.19 versus 8.89 ± 8.29 µg/ml, $p = 0.004921$, Figure 3). This pattern also held true when MIC_{90} values for all strains grown in complete media were pooled and compared (mean MIC_{90} 7.73 ± 6.65 versus 13.82 ± 7.86 µg/ml, $p = 2.4 \times 10^{-7}$), and when MIC_{90} values across all conditions were combined (mean MIC_{90} 5.63 ± 6.82 µg/ml versus 8.24 ± 7.62 µg/ml, $p = 0.0001887$, Figure 3).

***In vitro* cytotoxicity of dermaseptin derivatives against human gingival epithelial cells**. Peptide $K_4S_{4(1\text{-}15)}$ also performed better in cytotoxicity assays. More than 77% of cells survived growth with either AMP at concentrations equal to or below 1 µg/ml (77.1 ± 7.9%, Figure 4), but at higher concentrations $K_4K_{20}S_4$ became toxic. Only 22.3 ± 15.5% of cells survived 24 hour incubation with 4 µg/ml of $K_4K_{20}S_4$, and 6.03 ± 5.5% of cells survived incubation with 5 µg/ml (Figure 4). In contrast, more than two-thirds (69.4 ± 3.6%, Figure 4) of cells survived 24 hour incubation with 8 µg/ml $K_4S_{4(1\text{-}15)}$.

DISCUSSION

Long development timelines, coupled with expensive research and approval processes, drove more than ten large companies out of the novel antibiotic business between 1999 and 2004 (Projan and Shlaes 2004), and approvals for new drugs that kill or inhibit bacterial cells are increasingly rare (Ferri et al. 2017). This problem is compounded by the rise in antibiotic

resistant bacterial strains and infections (Boucher et al. 2009; Gierhart and Chukwuma 2017). New, effective treatments against drug resistant infections are urgently needed. One novel avenue for treating these difficult infections is with AMPs. These AMPs are positively charged small proteins with a high proportion of hydrophobic residues that force them into amphiphilic tertiary configurations (Pfalzgraff, Brandenburg, and Weindl 2018). Antimicrobial peptides can have bactericidal or bacteriostatic effects against a wide-spectrum of species (de la Fuente-Nunez et al. 2014). With their rapid bactericidal activity and easily engineered or modified structures, they are a promising family of compounds for combating the rise in antibiotic resistant infections and are less likely to induce the evolution of further bacterial resistance (Zairi et al. 2014). Recent studies show that AMPs can disrupt bacterial biofilms and are effective against both resistant, clinical isolates and laboratory strains (Lemon et al. 2018).

The dermaseptins are a superfamily of AMPs with antibacterial, antifungal, and antiviral activities (Nicolas and Ladram 2013). All dermaseptins share a conserved tryptophan residue at position 3 and an AA(A/G)KAAL(G/N)A consensus motif in their midregion (Nicolas and Ladram 2013). This motif enables the coil-to-helix transition that helps the AMP bind to lipid bilayers (Huang et al. 2017). This study investigated the antimicrobial activities of dermaseptin derivatives $K_4K_{20}S_4$, which has a double modification, with lysines at position 4 and 20, and the shorter, 15-mer version $K_4S_{4(1\text{-}15)}$. These lysines increase the net positive charge and reduces the hydrophobicity of the dermaseptin S_4, resulting in reduced hemolytic activity and making it an ideal AMP for *in vivo* applications (Navon-Venezia et al. 2002; Efron et al. 2002).

The two dermaseptin derivatives showed equivalent antibacterial activities against both drug-sensitive lab strains and MDR clinical isolates (Figure 3). These nondiscriminatory antibacterial activities, regardless of target bacteria drug resistance status, underpin a promising

new path to treat MDR infections. Though strains 105734 and 105765 resisted double- and triple-digit doses (in µg/ml) of ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftriaxone, cefuroxime, nitrofurantoin, piperacillin, tetracycline, rifampicin, vancomycin, clindamycin, clindamycin, cephalexin, minocycline, or linezolid (unpublished data), the bacteria were inhibited by 0.5 to 32 µg/ml of either AMP tested (Figure 3). $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$, therefore, could be strong, effective, and practical treatments for patients with MDR infections.

Though all Dermaseptins have some conserved characteristics, they can vary in in length, amino acid sequence, hydrophobicity, and charge (Nicolas and Ladram 2013). The MICs for many of these peptides are reported in previous literature, but these experiments were performed with varying conditions and protocols. Here we report that, independent of drug-resistant status, all *P. aeruginosa* strains tested were more resistant to dermaseptin derivatives when cultured in complete media (Figure 3). In some cases, the MIC of one AMP against bacteria cultured in complete media was eight times higher than when the bacteria were grown in minimal media. Media and growth conditions must be held constant to enable comparison of AMPs, including dermaseptins, across experiments.

Other groups previously tested dermaseptins for potential use in multiple areas, including as a topical application to treat oral infections (Porat et al. 2006), but the cytotoxic effects of dermaseptins on the gingival epithelium, an important mechanical barrier against bacterial invasion of the periodontal tissue (Fujita et al. 2018), has never been reported. We quantified the cytotoxicity of $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$ on HGEPs, and found $K_4K_{20}S_4$ has relatively higher cytotoxicity than $K_4S_{4(1\text{-}15)}$ (Figure 4). Because $K_4S_{4(1\text{-}15)}$ is not cytotoxic to HGEPs at doses below its mean MIC_{90} value (5.63 µg/ml, Figure 3) it could have practical use as a therapeutic tool.

$K_4K_{20}S_4$ is known for its higher antimicrobial activity and lower cytotoxicity than the original S_4 dermaseptin (Navon-Venezia et al. 2002). The shorter variant, $K_4S_{4(1\text{-}15)}$, is terminated before the AA(A/G)KAAL(G/N)A consensus motif, but the MIC value of $K_4S_{4(1\text{-}15)}$ was 32% lower than that of $K_4K_{20}S_4$ ($p < 0.001$). With its smaller size and correspondingly lower synthesis cost, lower cytotoxicity (Figure 4), and stronger antibacterial effects, $K_4S_{4(1\text{-}15)}$ is an even better candidate for treating MDR *P. aeruginosa* infections than the larger $K_4K_{20}S_4$.

MILITARY SIGNIFICANCE

We tested two dermaseptin derivatives for efficacy against *P. aeruginosa* laboratory strains and MDR clinical isolates. *P. aeruginosa* is relatively common, opportunistic, Gram-negative pathogen of concern to the U.S. military health system (Gierhart and Chukwuma 2017) and to the larger international medical community (Boucher et al. 2009). Other Gram-negative pathogens are also problematic in the military health system, but new antibiotics against Gram-negatives are especially rare (Ferri et al. 2017).

The antibiotics doripenem, imipenem, ticarcillin/clavulanic acid, ciprofloxacin, and levofloxacin are becoming less effective, especially in the military health system (Gierhart and Chukwuma 2017; Akers et al. 2014). That the two dermaseptin derivatives tested here, particularly the shorter variant $K_4S_{4(1\text{-}15)}$, are effective against antibiotic resistant clinical isolates of Gram-negative pathogens makes them excellent candidates for future development as bactericidals. Considering that both clinical strains used, *P. aeruginosa* isolates 105734 and 105765, have extremely high tolerance for most commercially available antibiotics including ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftriaxone, cefuroxime, nitrofurantoin,

piperacillin, tetracycline, rifampicin, vancomycin, clindamycin, clindamycin, cephalexin, minocycline, and linezolid (data not shown), their susceptibility to these AMPs is significant. Indeed, because new antibiotics against Gram-negative pathogens are rare, $K_4S_{4(1\text{-}15)}$, with its strong effects on *P. aeruginosa*, is an especially interesting antibacterial.

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FIGURES

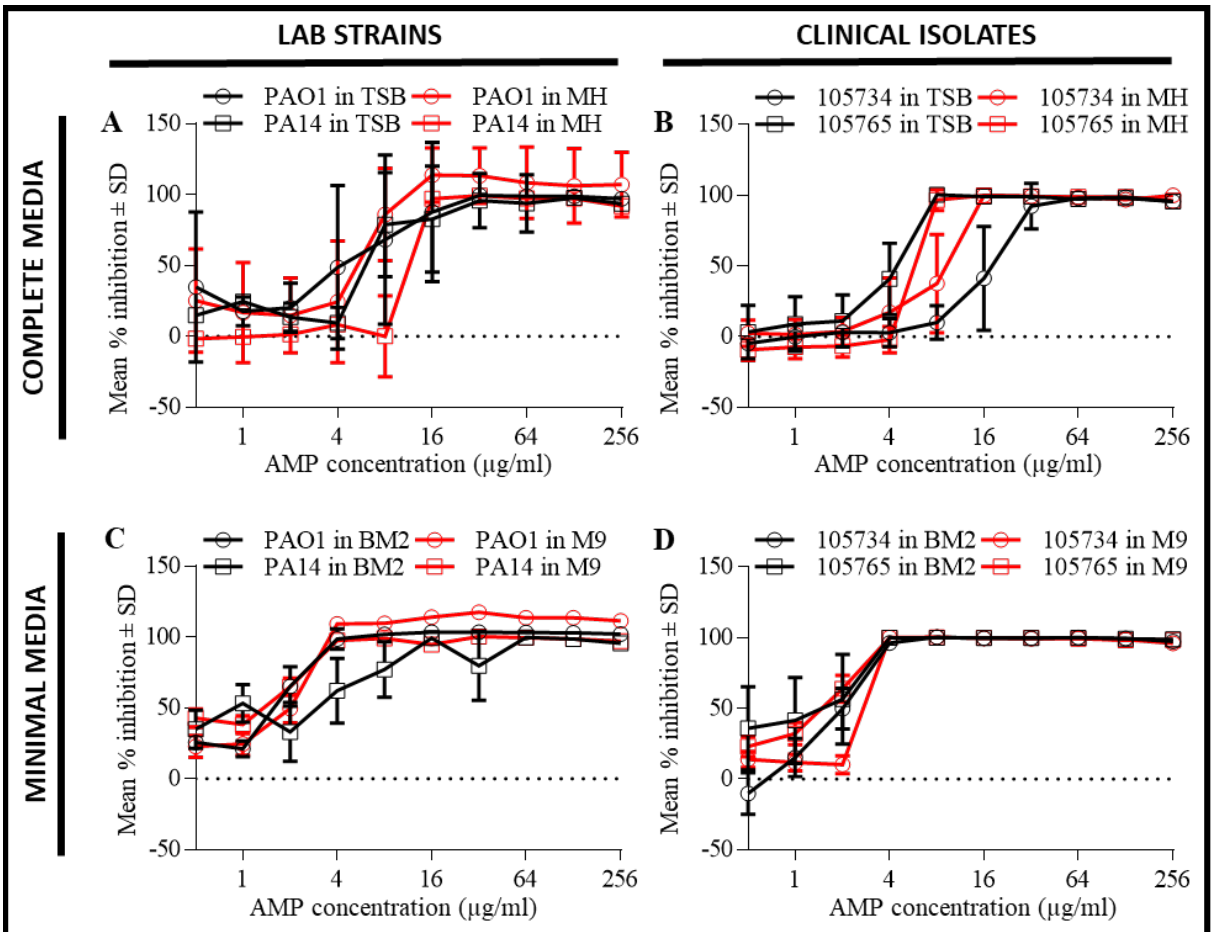

Figure 1. Antibacterial properties of $K_4K_{20}S_4$. Inhibition of bacterial growth varies with AMP concentration, *P. aeruginosa* strain type (laboratory versus clinical), and culture media (complete versus minimal). Mean inhibition of cultures grown in TSB and MH complete media shown in A and B, BM2 and M9 minimal media shown in C and D. Mean inhibition of laboratory strains PAO1 and PA14 shown in A and C, clinical strains 105734 and 105765 shown in B and D. Error bars represent one standard deviation from the mean.

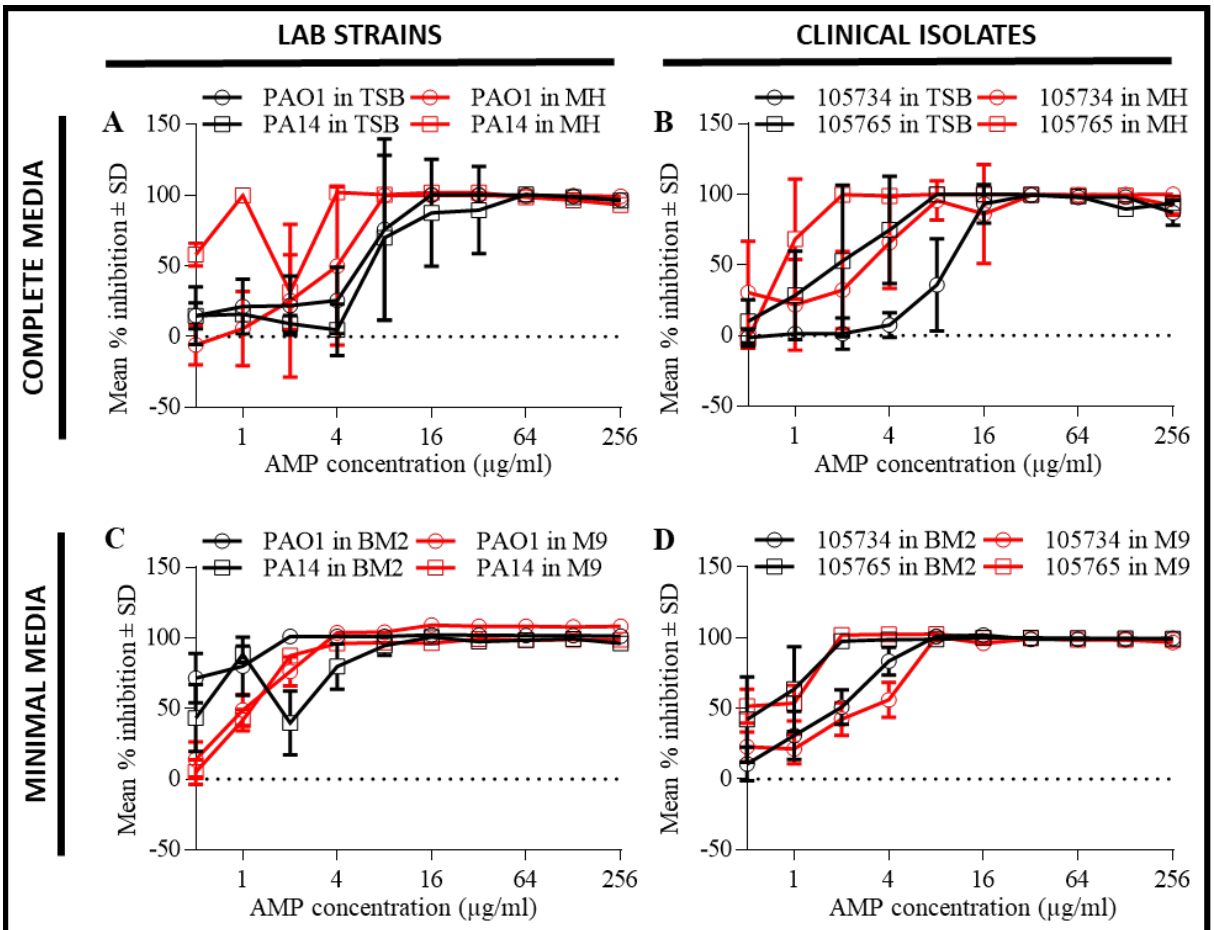

Figure 2. Antibacterial properties of $K_4S_{4(1\text{-}15)}$. Inhibition of bacterial growth varies with AMP concentration, *P. aeruginosa* strain type (laboratory versus clinical), and culture media (complete versus minimal). Mean inhibition of cultures grown in TSB and MH complete media shown in A and B, BM2 and M9 minimal media shown in C and D. Mean inhibition of laboratory strains PAO1 and PA14 shown in A and C, clinical strains 105734 and 105765 shown in B and D. Error bars represent one standard deviation from the mean.

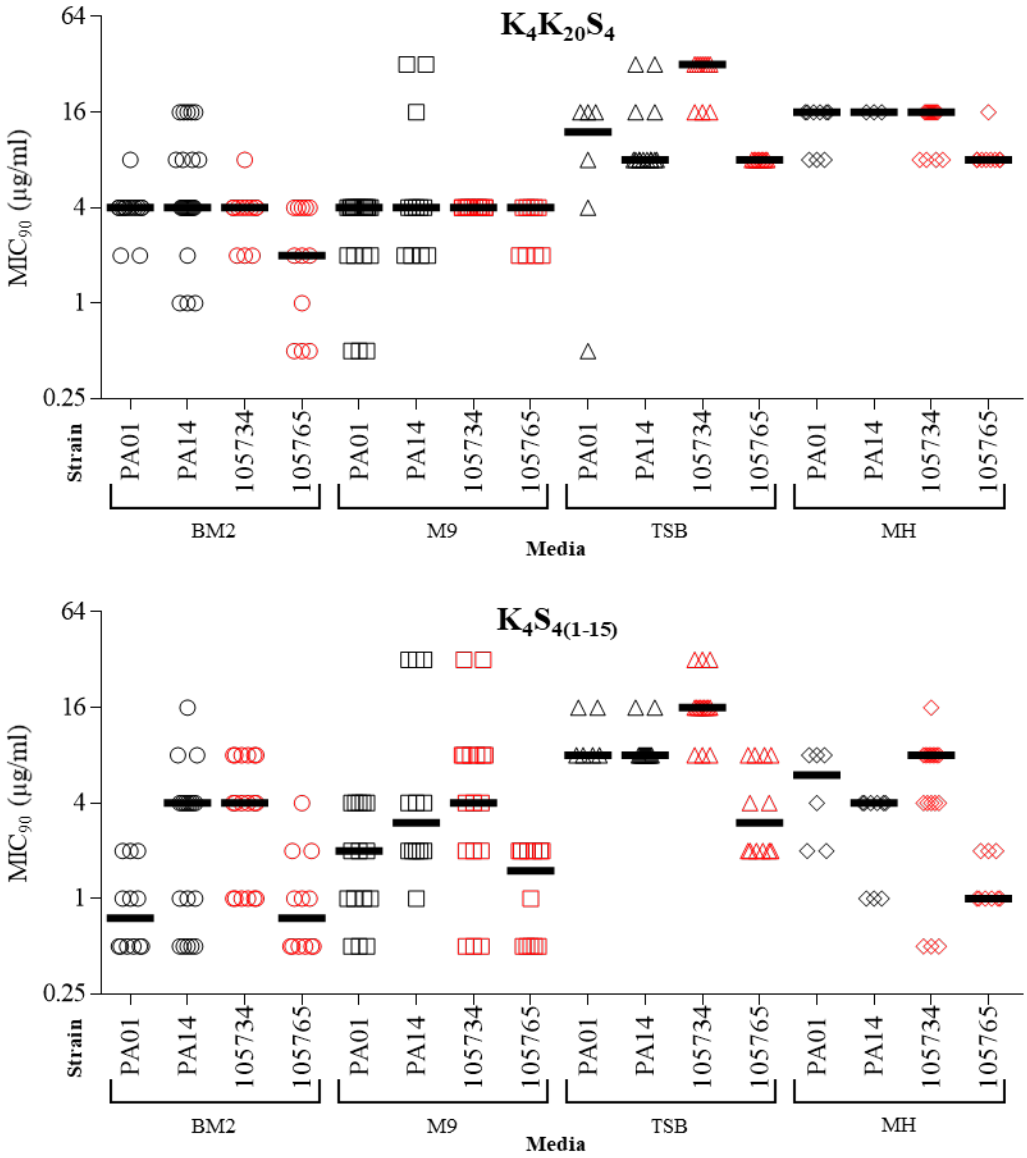

Figure 3. Minimum concentrations for bacterial growth inhibition. Minimum concentration to inhibit 90% of bacterial growth (MIC_{90}) for $K_4K_{20}S_4$ (top) and $K_4S_{4(1\text{-}15)}$ (bottom) for each combination of strain and media (x-axis). Black lines indicate median for each combination of *P. aeruginosa* laboratory (PAO1 and PA14) or clinical (105734 and 105765) strains and minimal (BM2 and M9) or complete (TSB and MH) media.

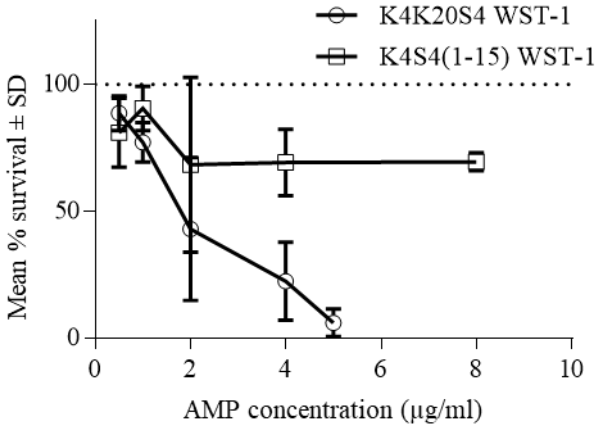

Figure 4. Cytotoxicity of $K_4K_{20}S_4$ and $K_4S_{4(1\text{-}15)}$. Mean percent survival of human gingival epithelial cells after 24 hours, as indicated by the cell proliferation reagent WST-1, varies with AMP concentration. Error bars represent one standard deviation from the mean.