A head-to-head comparison of the antimicrobial activities of 30 ultra-short antimicrobial peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*.

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Abstract

The rapid emergence of drug-resistant bacteria and the lack of new antibiotics entering the market is a major worldwide concern. Antimicrobial peptides are deemed plausible drug candidates as they specifically target and disrupt microbial cell membranes, causing death by cell lysis. However, their instability towards plasma proteases and perceived high manufacturing cost limit their potential for further drug development. A plausible solution is to identify and develop very short linear peptides as topical agents for treating skin and soft tissue infections. A literature survey yielded 30 ultra-short antimicrobial peptides up to 9 residues in length with antimicrobial activities. They were commercially synthesized and a head-to-head antimicrobial activity comparison was conducted on common skin pathogens including meticillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*. The topical broad-spectrum antibiotic Gentamicin and antifungal Nystatin were included as controls. Experimental results revealed only 2 peptides with potent broad-spectrum activities; octapeptide IRIRIRIR-NH_2 and nonapeptide Ac-KWRRWVRWI-NH_2 exhibited MICs of 6.25 μM against all test microbes. Both peptides have been reported to be non-cytotoxic to human cells, suggesting that they could potentially be further developed as topical antimicrobial agents.

Key Words

antimicrobial peptide; antibacterial peptide; skin infection; MRSA; Pseudomonas; Candida

1. Introduction

The emergence of multi-drug resistant bacteria has been increasing at alarming rates in both hospitals and community settings worldwide, posing a serious threat to human health (Andersson and Hughes, 2014; Boucher et al., 2009). In 2013, the Centers for Disease Control and Prevention (CDC) estimated at least 2 million people in the U.S. are infected with drug-resistant bacteria annually, resulting in more than 23,000 fatalities a year (www.cdc.gov/drugresistance/threat-report-2013/). Despite this, only 3 new antibiotic classes were introduced into the market over the past 13 years (2000–2013), resulting in a dire need for new antibiotics to overcome drug resistance mechanisms (Bassetti et al., 2013).

Antimicrobial peptides (AMPs) have generated considerable interest as substitutes for conventional antibiotics (Fjell et al., 2012; Hancock 2000; Yeung et al., 2011). Unlike conventional antibiotics that target specific microbial proteins or enzymes, AMPs target and physically disrupt microbial membranes, killing them by cell lysis (Wimley 2010). This membrane-targeting mechanism has been proposed to be an effective way to circumvent conventional mechanisms by which microbes become drug-resistant as developing resistance will likely require multiple mutations to change membrane morphology (Yeung et al., 2011). However, a major drawback of using AMPs as drugs is their susceptibility to proteolytic degradation by human proteases, limiting their routes of administration to topical and opthalmic applications (Fjell et al., 2012; Hancock 2000; Yeung et al., 2011). Hence, in our opinion, AMPs are best suited for development as topical agents for treating skin and soft tissue infections (SSTIs). Indeed, Omiganan, a 12-residue peptide analog of indolicidin isolated from bovine neutrophils is currently in phase 2 clinical trials as a topical agent for bacterial skin infections (http://clinicaltrials.gov/show/NCT01784133).

Another concern is the perceived high manufacturing cost of AMPs due to their relative large sizes compared to small-molecule drugs (Brogden and Brogden, 2011; Hancock, 2000; Hancock and Sahl, 2006). We believe this can be mitigated by identifying and selecting very short AMPs with potent antimicrobial activities for drug development. Using search terms 'short antibacterial peptide' and 'short antimicrobial peptide' in SciFinder® from the Medline database spanning 20 years (1993–2013) yielded 180 English language publications. Refining the search to linear peptides up to 9 natural amino acid residues in length revealed 30 peptides, herein termed 'ultra-short AMPs'. To identify the most potent AMP, all 30 peptides were synthesized commercially and subjected to a head-to-head minimum inhibitory concentrations (MICs) comparison against meticillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*, common human skin pathogens responsible for SSTIs (Dryden 2010; Hitchcock et al., 1987; Segal 2005). Their MICs were also compared to commercially-available topical broad-spectrum antibiotic Gentamicin, antifungal Nystatin and the experimental antibacterial peptide drug candidate Omiganan (Table 1).

Herein, we report the MICs of 30 ultra-short AMPs against common human skin pathogens to identify potential candidates for further drug development as topical agents for treating SSTIs.

2. Material and methods

2.1 Antibiotics, peptides and microbes

Gentamicin sulfate was purchased from Sigma-Aldrich (USA). Nystatin was purchased from Selleckchem (USA). Omiganan pentahydrochloride was purchased

from GL Biochem (China) and all other peptides were purchased from Mimotopes (Australia). All peptides were purified to ≥ 90% purity by HPLC. MRSA strains USA100 (ATCC-BAA-1681), USA300 (ATCC-BAA-1680), *Pseudomonas aeruginosa* (ATCC-CRM-9027) and *Candida albicans* (ATCC-90028) were purchased from ATCC (USA).

2.2 Antimicrobial assay

The MICs of antibiotics and peptides were determined using the microdilution method from the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2006). Briefly, bacteria were grown fresh from frozen stock in Mueller Hinton 2 agar while *C. albicans* was grown on yeast malt agar at 37 °C. After an overnight incubation, 5 MRSA and *P. aeruginosa* colonies were selected to grow in cation-adjusted Mueller Hinton broth 2 (Sigma-Aldrich #90922) while 5 *C. albicans* colonies were selected to grow in yeast malt broth (Sigma-Aldrich #Y3752) in a shaking incubator (220 RPM) at 37 °C. Cells were grown to an optical density (OD_{600}) of 0.15–0.16 using a Microplate Spectrophotometer (Molecular Devices Spectra Max Plus, USA), which corresponds to 1–2 × 10^8 CFU/mL. Test compounds were constituted into 4 mM DMSO stock solutions and then subjected to 2-fold serial dilution in a 96-well plate with concentrations ranging from 100 to 0.39 μM. 50 μL of microbial culture containing ~ 1–2 × 10^6 CFU/mL of microbes in the respective broths was introduced into each well containing 50 μL of compound. After an overnight incubation at 35°C, optical density measurements were conducted (OD_{600}) using the Microplate Spectrophotometer. All experiments were conducted twice independently in duplicates. The MIC was defined as the lowest antibiotic or peptide concentration (μM) required to inhibit microbial growth.

3. Results

The MICs of commercially-available topical antimicrobials, experimental drug candidate Omiganan and 30 ultra-short AMPs are tabulated in Table 1. Gentamicin, a topical broad-spectrum antibiotic, exhibited MICs of 0.39 μM against both strains of MRSA (USA100 and USA300) and 0.78 μM against *P. aeruginosa*. Expectedly, it was inactive against the fungi *C. albicans* (MIC >100 μM). Nystatin, a topical antifungal, exhibited an MIC of 3.125 μM against *C. albicans* while inactive against MRSA and *P. aeruginosa*. Omiganan exhibited MICs of 6.25 μM against both strains of MRSA but was relatively inactive towards *P. aeruginosa* and *C. albicans* (MICs 25 and 100 μM respectively).

[Insert Table 1 here]

Ultra-short AMPs constituting 3 and 4 residues (peptides **1**–**6**) were found to be inactive against all 4 microbes in Table 1 (MICs >100 μM). Hexapeptide **7** was found to be inactive against all test organisms while hexapeptides **8**–**10** exhibited a wide range of antibacterial activities on MRSA (MICs 12.5 to >100 μM), the most potent being peptide **10** with MICs of 12.5 μM against both MRSA strains. However, all hexapeptides were either inactive or weakly active against *P. aeruginosa* and *C. albicans* (MICs ≥50 μM).

Octapeptides **11**–**21** exhibited a wide range of antimicrobial activities against our panel of test pathogens. The most potent octapeptide, peptide **18**, exhibited broad-spectrum antimicrobial activities against MRSA, *P. aeruginosa* and *C. albicans* (MICs 6.25 μM). Interestingly, octapeptides **17** and **19** were found to be two-fold more

potent against *C. albicans* (MIC 1.56 μM) compared to the antifungal drug Nystatin (MIC 3.125 μM). In contrast, octapeptides **11**–**13** displayed either weak or no bioactivities against all 4 test pathogens (MICs ≥50 μM).

The longest peptides used in this study, nonapeptides **22**–**30**, exhibited mixed results. On one end, peptides **22**–**25** were either weakly active or inactive against all 4 pathogens (MICs ≥50 μM) while peptides **26** and **27** exhibited moderate activities against MRSA (MICs 12.5 μM). In contrast, nonapeptide **28**, like octapeptide **18**, exhibited broad-spectrum antimicrobial activities against all 4 microbes (MICs 6.25 μM). Lastly, nonapeptides **29** and **30** were found to be the most potent peptides against MRSA (MICs 3.125 μM) but were 8-fold less potent than Gentamicin (MIC 0.39 μM). Interestingly, nonapeptide **30** was also active against *P. aeruginosa* (MIC 3.125 μM) while nonapeptide **29** was relatively inactive (MIC 25 μM).

In summary, peptides with less than 8 residues lacked potent antimicrobial activities (MICs ≥12.5 μM) and only 5 out of the 30 peptides possessed potent activities against both MRSA strains (MICs ≤6.25 μM). Of these, only peptides **18** and **28** could be considered broad-spectrum antimicrobials as their bioactivities also covered *P. aeruginosa* and *C. albicans* (MICs 6.25 μM). The most potent peptides against MRSA were nonapeptides **29** and **30** (MICs 3.125 μM) but were inactive against the fungi *C. albicans* (MICs >100 μM).

4. Discussion

The human skin is the largest organ of the body and is consequently the most common target for microbial infections (Dryden 2010). Gram-positive *Staphylococcus aureus* is, by far, the most common pathogen responsible for SSTIs, accounting for more than 75% of reported cases in the U.S., of which close to 60% were found to be

meticillin-resistant (Moran et al., 2006). Two of the most common MRSA strains were thus selected for this study: USA100 and USA300, the most predominant hospital-acquired and community-acquired strains respectively (King et al., 2006; Limbago et al., 2009; Moran et al., 2006). Other common pathogens associated with SSTIs included in this study were Gram-negative *Pseudomonas aeruginosa* and the fungi *Candida albicans*, both responsible for approximately 11 and 8 % of SSTI cases in the U.S. respectively (Dryden 2010).

Due to the extensive use (and misuse) of antibiotics and coupled to the dearth of new antibiotics reaching the market, the emergence of multi-drug resistant bacteria and fungi have become a major medical concern worldwide, posing a dire threat to human health (Bassetti et al., 2013; Boucher et al., 2009.; Spellberg et al., 2008).

AMPs have generated considerable interest as substitutes for conventional antibiotics because they selectively target and physically disrupt microbial membranes, causing cell lysis and death (Fjell et al., 2012; Yeung et al., 2011). Their membrane-specific mechanism has been proposed to be an effective strategy to overcome conventional mechanisms by which microbial pathogens become drug-resistant (Fjell et al., 2012). However, a major hurdle in developing peptides as drugs is the perceived high manufacturing cost due to their large sizes as AMPs typically constitute ten or more amino acid residues (Brogden and Brogden, 2011; Hancock, 2000; Hancock and Sahl, 2006). To our best knowledge, the shortest linear AMP currently in clinical trials for skin infections is the 12-residue indolicidin-derived peptide Omiganan (ILRWPWWPWRRK-NH_2) (http://clinicaltrials.gov/show/NCT01784133). Hence, to identify shorter and possibly more potent AMPs as leads for antimicrobial drug development, 30 ultra-short AMPs

from the literature were commercially synthesized for a head-to-head MIC comparison against the aforementioned common skin pathogens (Table 1).

Results from Table 1 revealed that the topical broad-spectrum antibiotic Gentamicin, an aminoglycoside targeting bacterial 30S ribosomal subunit involved in protein synthesis, was highly potent against MRSA and *P. aeruginosa* (MICs 0.39 and 0.78 μM respectively). However, reports on the emergence of Gentamicin-resistant MRSA in European hospitals (Pérez-Vázquez et al., 2009; Schmitz et al., 1999], coupled to its ototoxic and nephrotoxic side-effects (Selby et al., 2009), highlight the urgent need for new antibiotics. The macrocycle Nystatin targets ergosterol in fungal cell membranes to form transmembrane pores, causing cell death. As a topical antifungal, Nystatin was found to be active against *C. albicans* in our assay (MIC 3.125 μM) and was inactive on MRSA and *P. aeruginosa* as expected (Table 1). A report on Nystatin-resistant *Candida* first appeared in 1971 (Athar and Winner 1971; Hitchcock et al., 1987), emphasizing the need for new antifungals for *Candida* infections including cutaneous, oral and vaginal candidiasis. Omiganan, a 12-residue indolicidin derivative currently in phase 2 clinical trials as a topical bactericidal for the treatment of Gram-positive SSTIs, exhibited potent activities against MRSA (MIC 6.25 μM), moderate activity against *P. aeruginosa* (MIC 25 μM) and insignificant activity against *C. albicans* (MIC 100 μM), in agreement with the literature (Sader et al., 2004).

MICs of the 30 peptides in Table 1 suggested some correlation between antimicrobial potencies and peptide length. For example, peptides with 4 or less residues were found to be inactive, contrary to the literature reports referenced in Table 1. Peptide **1** (GHK-OH) is a tripeptide found in human plasma with antibacterial activity against an unspecified strain of *S. aureus*. Its MIC was not reported. Peptide **2**

(Ac-KPV-NH_2) is a tripeptide fragment from α-melanocyte stimulating hormone secreted by pituitary cells with reported antimicrobial activities against unspecified clinical isolates of *S. aureus* and *C. albicans* from Italy. Its MIC, however, was not revealed. Peptide **3** (WFN-OH) is a tripeptide with reported antibacterial activities against laboratory strains of *S. aureus* (NCIM 2079) and *P. aeruginosa* (NCIM 2036) from India. MIC values were not reported. Peptide **4** (KYR-OH) is a tripeptide fragment from bovine haemoglobin α-chain with a reported MIC of 1 μM against an unspecified strain of *S. aureus* isolated from food products in France. Peptide **5** (RYH-OH) is a tripeptide fragment from bovine haemoglobin β-chain with reported MICs of 1 μM against *Salmonella*, *Listeria innocua* and *Micrococcus luteus* isolated from food products in France. Peptide **6** (Ac-OOWW-NH_2) is a rationally-designed tetrapeptide containing two ornithines with a reported MIC of 2 μg/mL (~3 μM) against *S. aureus* MTCCB-96 from the Microbial Type Culture Collection in India. Our assay results revealed peptides **1**–**6** were inactive towards our panel of skin pathogens (MICs >100 μM). A reason could be that they were too short to physically disrupt the microbial membranes and/or could be due to the different bacteria and fungi strains used in our study.

Peptide **7** (KWKWKW-OH) is a rationally-designed synthetic hexapeptide with reported MICs of 32 and 16 μM against *P. aeruginosa* KCTC-1637 and *C. albicans* KCTC-7270 respectively from the Korean Collection for Type Culture (KCTC). Tryptophan-rich peptide **8** (WRWRWR-NH_2) is a rationally-designed synthetic tryptophan-rich hexapeptide with reported MICs of 7.7 and 15.3 μM against *S. aureus* KCTC-1621 and *P. aeruginosa* KCTC-1637 respectively. Our assay results revealed peptides **7** and **8** to be either inactive or relatively inactive against both MRSA strains (MICs ≥25 μM) and were also inactive against *P. aeruginosa* and *C. albicans* (MICs

>100 μM). Tryptophan-rich peptides **9** and **10** (RRRWWW-NH_2 and RWRWRW-NH_2) are rationally-designed synthetic hexapeptides with reported MICs of 5 μg/mL (~4.7 μM) against MRSA ATCC-33591. Our experimental results for MRSA USA100 and USA300 ranged from 12.5–25 μM, suggesting that some MRSA strains were more susceptible to AMPs. A check in the ATCC website (www.atcc.org) revealed that the ATCC-33591 strain reported in the literature was a clinical isolate from the 1970s while the USA100 and USA300 strains used in our experiments were 2003 clinical nasal and skin isolates respectively which may possess thicker cell membranes or walls to hinder the bactericidal effect of AMPs. We also noted that hexapeptides **9** and **10** possessed the same net number of positive charges (+4) but exhibited a two-fold difference in potencies against MRSA, suggesting that the relative positioning of R and W residues in a peptide was an important determinant of antimicrobial activity. Also noteworthy were hexpeptides **8** and **10** (WRWRWR-NH_2 and RWRWRW-NH_2 respectively) whose sequences were reversed. The latter was found to be 2-fold more potent than the former (12.5 vs. 25 μM respectively) using our MRSA panel. At this point in time, we are unable to provide a plausible explanation for this and suggest membrane-perturbation studies using molecular dynamics simulations to be conducted in future to shed further insights.

Peptide **11** (RKLKHMRF-OH) is an octapeptide fragment from secretogranin II, a neuroendocrine secretory protein secreted by bovine Chromaffin cells with a reported MIC of 5 μM against *Micrococcus luteus*. Peptide **12** (PFKISIHL-NH_2) is an octapeptide isolated from royal jelly with reported bactericidal activity on clinical *S. aureus* isolate A-170 from Italy. Peptide **13** (FFFLSRIF-NH_2), also known as Temporin-SHf, is an octapeptide isolated from the skin of the Sahara frog *Pelophylax saharica*. It was touted to be the shortest linear AMP reported to date with an MIC of

12.5 μM against meticillin-susceptible *S. aureus* (MSSA) ATCC-25923. However, our assay results revealed peptides **11**–**13** to be weakly active or inactive (MICs ≥50 μM) towards our panel of skin pathogens (Table 1). A possible explanation for their lack of or weak antimicrobial activities could be due to the negatively-charged C-terminus of **11** and the low net positive-charge (+2) of **12** and **13**. In contrast, octapeptides **14**–**21** possessed high net positive-charges (≥ +4), exhibiting MICs of ≤25 μM against both MRSA strains. This observation is consistent with the literature as it has been postulated that AMPs must first bind to the anionic bacterial membrane before exerting their bactericidal effect (Wimley, 2010).

Peptide **14** (RRWYRWWR-NH_2) is a rationally-designed synthetic octapeptide with MIC of 1.8 μM against MSSA ATCC-25923. Peptide **15** (KWKWWKWK-NH_2), also known as Tet112, is an octapeptide identified from a peptide screening library with MSSA ATCC-25923 antibacterial activity. Its MIC, however, was not reported. Peptide **16** (RWRWRWRW-NH_2) is a rationally-designed tryptophan-rich synthetic octapeptide with a reported MIC of 5.1 μM against MRSA ATCC-BAA-44. Our MIC results for peptides **14**–**16** were found to be in the range of 6.25–12.5 μM for MRSA USA100 and USA300, comparable to the reported results for peptide **16** (Liu et al., 2007) but were about 4-fold less potent compared to those reported for peptide **14** against MSSA ATCC-25923 (MIC 1.8 μM) (Strøm et al., 2002). A plausible explanation could be due to morphological differences in the cell membranes of MSSA and MRSA.

Peptides **17**–**19** are rationally-designed β-sheet forming octapeptides with alternate isoleucine and cationic residues. Of these, peptide **18** (IRIRIRIR-NH_2) exhibited potent wide-spectrum antimicrobial activity against *S. aureus* ATCC-29737, *P. aeruginosa* ATCC-CRM-9027 and *C. albicans* ATCC-10231 with MICs of 7.8–15.6

mg/L (~7–13 μM) respectively (Ong et al., 2013). This was in general agreement with our results showing MICs of 6.25 μM for all 4 microbes in Table 1. Peptide **18** possessed an interesting feature: it contained only two types of residues: isoleucine and arginine. When considered together with its short length (8 residues), the cost of manufacturing this peptide should be significantly lower compared to the 12-residue Omiganan.

Peptides **20** and **21** (RIWVIRWR-NH_2 and RIWVIWRR-NH_2) are antibacterial linear nonapeptides modified from the cyclic antimicrobial peptide Bectenecin from bovine neutrophils (Hilpert et al., 2005). Known as Bac8b and Bac8c respectively, both were potent against MSSA ATCC-25923 with MICs of 2–4 μg/mL (~2–4 μM). Our assay results on MRSA revealed MICs of 6.25–12.5 μM, again suggesting that MSSA was more susceptible to AMPs compared to MRSA. Bac8b and Bac8c were also reported to be active on *P. aeruginosa* H103 with MICs between 8–16 μg/mL (~7–14 μM). Our experiments on *P. aeruginosa* ATCC-CRM-9027 revealed MICs of 12.5–25 μM (Table 1), similar to the literature (Hilpert et al., 2005). Also noteworthy was the sequence similarity of **20** and **21** where the C-terminal sequence RWR was switched to WRR, resulting in a 2-fold potency improvement against both test MRSA strains and *P. aeruginosa* for **21** (Table 1). As both peptides were of the same length and possessed the same overall net charge (+4), our MIC results suggested that the placement of two consecutive arginines at the C-terminus was beneficial for antimicrobial activity. The same phenomenon was also observed in the original literature report on MSSA ATCC-25293 (Hilpert et al., 2005).

The last series of peptides (**22**–**30**) were nonapeptides which exhibited vastly different antimicrobial activities in our assays. Peptide **22** was selected from a 16,384-member combinatorial peptide library after a lipid vesicle permeability screen. It was

reported to have broad-spectrum activity against MSSA ATCC-25923 and *P. aeruginosa* ATCC-27853 with MICs of 3 and 2 μM respectively (Rathinakumar et al., 2009). Peptide **23** (RLWLAIKRR-NH_2) is a truncated nonapeptide analog of protaetiamycine, an insect defensin isolated from beetle larvae. Its reported MICs on *S. aureus* KCTC-1621 and *P. aeruginosa* KCTC-1637 were 2 and 8 μM respectively (Shin et al., 2009). Peptide **24** (KKKKKKKKK-NH_2) was shown to inhibit the growth of MSSA ATCC-25923 during a screen of synthetic homopeptides. Unfortunately, MICs were not reported (Guzmán et al., 2013). Peptide **25** (KLKLLLKLK-NH_2) is a truncated nonapeptide analog of sapecin B, an antimicrobial peptide isolated from the flesh fly *Sarcophaga peregrina*, with a literature MIC of 2 μM against MRSA SPM 101 (Naidoo and Rautenbach, 2013). In our assays, peptides **22–24** were inactive on all 4 test pathogens (MICs >100 μM) while peptide **25** was found to be weakly active against both test MRSA strains (MICs 50 μM) although it exhibited moderate activity against *P. aeruginosa* (MIC 12.5 μM). We postulated that the discrepancies in MICs to be due to the different microbial strains used. Particularly noteworthy was that peptide **25**, with a net positive-charge of +10, was found to be inactive against all test microbes (MICs >100 μM), suggesting that introducing an excessive number of cationic residues to a peptide may be detrimental to its antimicrobial properties. In contrast, peptide **22** with only one cationic residue and a net +2 charge also lacked bioactivity against our test pathogens, suggesting that having a low number of positive-charges may also be detrimental to a peptide's antimicrobial properties. Peptide **26** (PFWRIRIRR-NH_2) is a truncated Lactoferrin nonapeptide derivative with unreported antibacterial activity against *E. coli* NCTC 8007 from Spain (Zorko et al., 2009). Our assay results showed peptide **26** to be moderately active on both MRSA strains (MICs 12.5 μM) but inactive against *P. aeruginosa* and *C. albicans* (MICs ≥100 μM).

Peptide **27** (WKWLKKWIK-NH_2) is a nonapeptide identified from a peptide screening library with potent bioactivities against *Mycobacterium tuberculosis* (MIC 1.1 μM), MSSA ATTC-25923 (MIC 0.7 μM) and *P. aeruginosa* H103 (MIC 2.9 μM) (Ramón-García et al., 2013). Our experimental results showed it to be moderately active against MRSA and *P. aeruginosa* (MICs 12.5 μM). Peptide **28** (Ac-KWRRWVRWI-NH_2), also known as Pac-525, is a nonapeptide identified from screening a tryptophan-rich combinatorial peptide library with broad-spectrum activities against MSSA ATCC-29213 and *E. coli* ATCC-25922 with MICs of 4 and 2 μM respectively (Wei et al., 2006). Tryptophan is known to possess a high membrane-insertion propensity, facilitating tryptophan-rich peptides to partition into and disrupt membranes (Fimland et al., 2002). This was in agreement with our experimental results showing peptide **28** to be potent against MRSA, *P. aeruginosa* and *C. albicans* with MICs of 6.25 μM (Table 1). Interestingly, the shorter tryptophan-rich hexapeptide **7** was devoid of antimicrobial activity against our panel of pathogens (MICs >100 μM) while tryptophan-rich hexapeptides **8** and **9** were found relatively inactive against both test MRSA strains (MICs 25 μM) and were either weakly active or inactive against *P. aeruginosa* and *C. albicans* (MICs ≥50 μM), suggesting that peptide length and the relative positioning of arginine and tryptophan residues in a peptide sequence played an important role in conferring antimicrobial activities to tryptophan-rich peptides. In contrast, the high net charge (+5) tryptophan-rich octapeptides **14–16** were found to be active against MRSA (MICs 6.25–12.5 μM) but were either weakly active or inactive against *P. aeruginosa* and *C. albicans* (MICs ≥50 μM), suggesting that: (i) 9 residues was the minimum length required for broad-spectrum antimicrobial activity for tryptophan-rich peptides and (ii) having a high net charge did not guarantee the conferment of broad-spectrum activities. The relative positioning of tryptophan

residues was also important for *C. albicans* bioactivity. For example, nonapeptides **27** and **28** each possessed 3 tryptophan residues but the former was found inactive against *C. albicans* (MIC >100 μM) whereas peptide **28** was found to be potent (MIC 6.25 μM). Another difference between them was that the cationic residues in **27** consisted solely of lysines while those of **28** were mainly arginines, suggesting that the molecular structure of cationic residues also played a role in conferring anti-fungal activity.

Peptide **29** (KRWWKWWRR-NH_2), also known as Tet127, is a nonapeptide identified from a cellulose-tethered peptide screening library based on cathelicidins, antimicrobial peptides isolated from leukocytes (Zanetti, 2004). In its free form, peptide **29** was reportedly highly potent against *P. aeruginosa* PAO1 (MIC 0.7 μg/mL, ~0.5 μM) (Hilpert et al., 2009). In contrast, our assays revealed peptide **29** to be potent against both MRSA strains (MICs 3.125 μM) while only moderately active towards *P. aeruginosa* ATCC-CRM-9027 (MIC 25 μM). A plausible explanation could be due to the different strain of *P. aeruginosa* used in our study.

Finally, peptide **30** (KWRRWIRWL-NH_2), also known as P9-4, is a rationally-designed synthetic nonapeptide similar to Pac-525 (peptide **28**) with reported broad-spectrum activities against MSSA ATCC-6538 (MIC 3.1 μg/mL, ~2 μM), *P. aeruginosa* ATCC-CRM-9027 (MIC 6.2 μg/mL, ~4 μM) and *C. albicans* ATCC-10231 (MIC 3.1 μg/mL, ~2 μM) (Qi et al., 2010). Our experimental results revealed MICs of 3.125 μM for MRSA and *P. aeruginosa* ATCC-CRM-9027 but no activity against *C. albicans* ATCC-90028 (MIC >100 μM). A possible reason could be due to the different *C. albicans* strains used: ATCC-10231 was a clinical respiratory tract isolate while ATCC-90028 used in our assay was isolated from human blood and could thus possess different cell membrane morphologies.

In summary, octapeptide **18** and nonapeptide **28** were found to possess broad-spectrum activities on all 4 pathogens (MICs 6.25 μM), including the fungi *C. albicans*. Constituting 8 and 9 residues respectively, they were 25–33% shorter than the12-residue experimental drug candidate Omiganan while exhibiting comparable potencies against MRSA (MICs 6.25 μM) and were also active against Gram-negative bacteria and fungi (MICs 6.25 μM). Also noteworthy was peptide **30**, the most potent AMP against MRSA and *P. aeruginosa*. Also constituting 9 residues, it was 25% shorter than Omiganan but exhibited a 2-fold potency enhancement against MRSA (MIC 3.125 vs. 6.25 μM) and equipotent against *P. aeruginosa*. This suggested that with proper peptide sequence modification, it was possible to design ultra-short peptides with improved antimicrobial activities. This could lead to increased drug efficacy while lowering manufacturing cost. Peptides **18**, **28** and **30** were also reported to be non-toxic towards human cells (Ong et al., 2013; Qi et al., 2010; Wei et al., 2006), making them plausible candidates for further drug development as topical agents for treating SSTIs.

Acknowledgements

We thank A*STAR Biomedical Research Council for financial support.

Conflict of Interest

Qiu Ying Lau, Xing Yao Choo, Zhi Xue Lim, Xin Ni Kong, Fui Mee Ng, Melgious J. Y. Ang, Jeffrey Hill and C. S. Brian Chia declare that they have no conflict of interest.

Statement of human and animal rights and informed consent

This article does not contain any studies involving human or animal subjects performed by any of the authors.

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Captions

Table 1. In-house experimental MICs (μM) of commercially synthesized ultra-short antimicrobial peptides against various skin pathogens. Cationic residues are denoted in red. References refer to the first published report of the corresponding peptide.