Synthetic Antibacterial Peptide Exhibits Synergy with Oxacillin against MRSA

John C. Lainson,[†] Seth M. Daly,[‡] Kathleen Triplett,[‡] Stephen Albert Johnston,[†] Pamela R. Hall,[‡] and Chris W. Diehnelt^{*,†}

[†]Biodesign Institute Center for Innovations in Medicine, Arizona State University, Tempe, Arizona 85281, United States

[‡]University of New Mexico College of Pharmacy, Albuquerque, New Mexico 87131, United States

S Supporting Information

ABSTRACT: One proposed solution to the crisis of antimicrobial resistant (AMR) infections is the development of molecules that potentiate the activity of antibiotics for AMR bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Rather than develop broad spectrum compounds, we developed a peptide that could potentiate the activity of a narrow spectrum antibiotic, oxacillin. In this way, the combination treatment could narrowly target the resistant pathogen and limit impact on host flora. We developed a peptide, ASU014, composed of a *S. aureus* binding peptide and a *S. aureus* inhibitory peptide conjugated to a branched peptide scaffold, which has modest activity against *S. aureus* but exhibits synergy with oxacillin for MRSA both *in vitro* and in a MRSA skin infection model. The low concentration of ASU014 and sub-MIC concentration of oxacillin necessary for activity suggest that this molecule is a candidate for future medicinal chemistry optimization.

KEYWORDS: MRSA, antibacterial peptide, oxacillin, potentiator

Antimicrobial resistance (AMR) is a global crisis with few solutions on the horizon.¹ In the United States, resistant infections account for over 2,000,000 illnesses each year with MRSA estimated to be directly responsible for over 11,000 deaths per year.² *S. aureus* has multiple intrinsic and acquired resistance mechanisms that demonstrate the challenges of developing antibiotics that are both efficacious and slow to select for resistance. A number of strategies have been proposed to address this challenge, including (i) developing narrow spectrum antibiotics that limit activity against commensal bacteria or nontargeted pathogens^{3,4} and (ii) developing antibiotics that have multiple targets and/or a complex mechanism-of-action (MOA). Antibiotics with multiple targets have a high fitness cost associated with resistance and resistance generally emerges more slowly.⁵ The lipopeptide antibiotic, daptomycin, is a good example of a narrow spectrum *S. aureus* antibiotic with a complex MOA that, despite many years of clinical use, has been selected for resistance at a slow rate.⁵ Daptomycin was recently shown to have a complex MOA in which it inhibits cell envelope synthesis through a gradual depolarization of the membrane leading to a rearrangement of fluid lipid domains that displaces proteins involved in cell wall synthesis.⁶ Additionally, it has been shown that daptomycin works in concert with cell-wall-targeting β -lactam antibiotics to effectively treat MRSA infections.⁵ However, daptomycin was originally discovered in the late 1980s, and there are few platforms for discovering new antibacterials with similar MOAs.⁷

Antimicrobial peptides (AMPs), due to their membrane targeting action, offer a possible source of molecules that can potentiate the action of a number of antibiotics.^{8,9} Additionally, other cationic peptide mimics have demonstrated potentiation of multiple antibiotics.¹⁰ However, AMPs have a number of major unresolved technical challenges on the path to becoming a source of new antibiotics. First, there are limited numbers of naturally occurring and synthetic AMPs. For example, there are only 2786 AMPs listed in the most recent version of the Antimicrobial Peptide Database.¹¹ Second, AMPs are poor single agent antibiotics due to low activity, high toxicity, and poor drug-like properties.¹² AMPs can function as β -lactam potentiating molecules at sub-MIC concentrations; however, these concentrations are still higher than desired for a systemic therapeutic. Therefore, optimization of lead AMPs is the critical step in AMP drug development. Medicinal chemistry optimization can be difficult as many AMPs rely on secondary structure for activity, which can be disrupted by amino acid substitution. It is widely known that D-amino acid substitution can increase protease resistance, but the change in stereochemistry often has unpredictable effects on activity and toxicity.¹³ Other noncanonical amino acids offer similar protease stability improvements, but generally at a significantly higher cost, an important limitation of this strategy for the price-sensitive antibiotic market. While multimerization has

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been used to improve AMP activity,^{14–19} most efforts have focused on using synthetic methods for the multimerization of a single AMP. While not a true dimeric AMP, as it is prepared as a single linear peptide sequence, a broad spectrum, dual-targeted AMP composed of a region that targets *Streptococcus mutans* is currently in a Phase 2 clinical trial for dental caries.²⁰ This molecule has demonstrated high pathogen specificity, which opens the door to targeted killing without disturbing the host microbiome,²⁰ another potential advantage of narrow spectrum antibiotics.³ Finally, many AMPs are sensitive to ionic strength, buffer composition, and competing proteins and have a poor rate of translation of *in vitro* activity into *in vivo* function.¹²

Our group has established a discovery system to develop bivalent peptides, called synbodies, that inhibit bacterial growth.²¹ Our goal has been to use this system to produce synbodies with narrow spectrum activity. Our initial work produced a synbody, **ASU001**, that inhibited the growth of *S. aureus* with a minimum inhibitory concentration (MIC) of 14 μM and similar MICs against *Bacillus subtilis* and *Pseudomonas aeruginosa*.²¹ The synbody was composed of a peptide that inhibited bacterial growth (RWRRHKHFkRPHRKHkRGSC, **pep1**) and a second peptide that bound *S. aureus* without inhibiting growth.²¹ However, these peptides were composed of L-amino acids that were sensitive to protease degradation. We developed variants of **pep1** in which either Lys or both Lys and Arg were replaced with their D-enantiomers (indicated below by lowercase single amino acid abbreviation) and then conjugated to a bivalent maleimide scaffold.²² The synbody composed of two copies of the D-Arg, D-Lys substituted peptides, **ASU008**, maintained activity against MRSA after 6 h of incubation in fresh sera, demonstrating that **ASU001** could be modified to improve stability and activity using a combination of D-amino acids and bivalent scaffolds.

We have developed an additional variant of **ASU001** with improved *S. aureus* specificity by selecting a new *S. aureus* binding peptide, **p147**, and incorporating it along with a D-Lys substituted version of **pep1**, referred to as **D-Ly**, to produce a new synbody, **ASU014**. We used maleimide–thiol conjugation²² to couple both peptides (denoted as $R_{1/2}$ as we do not control which peptide is conjugated to which maleimide group) to a bivalent scaffold that contains an additional peptide (R_2) to simulate synbody conjugation to a carrier protein or peptide that might be used for half-life extension (Figure 1).²³ We then tested the *in vitro* activity against *S. aureus*, *P. aeruginosa*, and *Escherichia coli* as well as function in an air pouch model of MRSA infection. Here we describe identification of the lead synbody, **ASU014**, and demonstrate its synergy with oxacillin both *in vitro* and in the air pouch model of infection.

To produce a synbody with a more narrow spectrum of activity than **ASU001**, we set out to identify new *S. aureus* binding peptides that could be conjugated to **D-Ly**. To identify a new *S. aureus* binding peptide, we screened a methicillin-sensitive *S. aureus* (MSSA) strain and *P. aeruginosa* PAO1 against a library of 10,000 peptides, 20 amino acids in length, that was immobilized on a peptide microarray²⁴ using our previous dual label approach.²¹ From this screen we selected three peptides to use for synbody construction (Table S1). Peptides were chosen based upon MSSA binding with low *P. aeruginosa* binding. We selected a high (**p43**), medium (**p147**), and low (**p91**) MSSA binder for use in synbody construction. Each peptide was synthesized (Sigma Custom Peptide, The Woodlands, TX, USA) and did not inhibit MSSA growth when

Figure 1. Structure of **ASU014**.

tested at 50 μM . We then prepared two possible *S. aureus* specific synbodies: **ASU010**, the heterobivalent combination of **p43** and **D-Ly** ([Ac]-RWRRHKHFkRPHRKHkRGSC), and **ASU014**, the combination of **p147** and **D-Ly**, through conjugation to a bis-maleimide scaffold (Sc2a) using previously developed conjugation methods (Figure S1).^{22,23} We used the Sc2a scaffold as it contains an additional 20mer peptide (R_2 = IMKPFETHRLGPERFDGSG) to both increase the molecular weight of the synbodies above the kidney filtration threshold and to simulate conjugation to another half-life extending peptide or carrier protein. We also prepared a negative control synbody, **ASU042**, composed of two copies of **p91** on Sc2 (Figure S2). Each synbody was purified and tested for growth inhibition of MSSA, with **ASU014** inhibiting growth after overnight incubation at 50 μM , while **ASU010** had minimal effect on MSSA growth (Figure S3). We evaluated synbodies composed of two copies of **p147** and found no growth inhibition (data not shown), while the synbody composed of two copies of D-Lys was not pursued due to its high cationic charge. We measured the MIC of **ASU014** against MSSA, MRSA, *P. aeruginosa*, and *E. coli* O157:H7 and found that it had MICs that ranged from 25 to 100 μM for *S. aureus* and no inhibition against the Gram-negative bacteria (Table 1, Table S2). **ASU014** was tested for toxicity against mouse red blood cells and exhibited no hemolytic activity at 500 μM . We did not test **ASU014** at concentrations higher than 500 μM due to reduced solubility at concentrations >500 μM .

Table 1. **ASU014** Minimum Inhibitory Concentration (MIC)

species	MIC (μM)
MSSA	100
MRSA-USA100	100
MRSA-USA300-HI168	100
MRSA-USA300-LAC	25
<i>P. aeruginosa</i>	n.i. ^a
<i>E. coli</i> O157:H7	n.i. ^a
hemolysis at 500 μM	<10%

^an.i. = no inhibition.

We then examined the bactericidal activity of ASU014 toward the current epidemic MRSA strain USA300-LAC²⁵ and found that ASU014 was bactericidal ($>3 \log_{10}$ reduction) at 25 μM (Figure S4). A second multidrug resistant USA300 strain (HI-168) was tested in a time-kill kinetic study, and while ASU014 was rapidly bactericidal at 100 μM , it loses activity with longer incubation (Figure 2A). We then tested ASU014

Figure 2. ASU014 kills USA300 MRSA and is synergistic with oxacillin. (A) Time-kill kinetics of ASU014 versus USA300-HI168 (circles). The synbody was added at 100 μM (squares), 50 μM (triangles), 25 μM (open squares), or 12.5 μM (open triangles) and incubated for the indicated time. (B) Isobologram of ASU014 with Oxa for USA300-LAC. ASU014 MIC alone (red), Oxa alone (blue), and combination (black) are shown. Graph is the average of replicate experiments.

for synergy with oxacillin (Oxa) using a checkerboard assay against USA300-LAC and found that ASU014 is synergistic with Oxa. This is demonstrated in the isobologram (Figure 2B) where the MIC as a function of ASU014 and Oxa concentration is shown. Combinations that are additive would fall on a diagonal line from the ASU014 MIC to the Oxa MIC, while combinations that are synergistic fall below that line. Additional testing of ASU014 with Oxa against a highly resistant hospital-acquired MRSA USA100 strain and the USA300-HI68 MRSA strain found that ASU014 reduced the Oxa MIC 16-fold (Table 2). This yielded fractional inhibitory concentration (FIC) values of FIC = 0.25 (USA300-LAC) and 0.19 (USA100). To confirm that this effect was not unique to oxacillin, we repeated the assay using nafcillin, another narrow-spectrum, penicillinase resistant β -lactam, and found that ASU014 was synergistic with nafcillin (FIC = 0.19) for USA100 but only additive for USA300-HI168 (Table 2). When we tested synergy with oxacillin on a MSSA strain, we observed an additive rather than a synergistic effect. As the USA100 strain is multidrug resistant, we investigated ASU014 synergy with other antibiotics and found that it was synergistic with amoxicillin, but not tetracycline or streptomycin (Table S3). These data suggest that ASU014 selectively potentiates the activity of β -lactams against multiple MRSA isolates.

Table 2. ASU014 Synergy with β -Lactam Antibiotics

	MIC ($\mu\text{g/mL}$)	combination [$(\mu\text{g/mL})/(\mu\text{M})$]	FIC	antibiotic fold change
Oxacillin				
MRSA USA100	128	8/12.5	0.19	16
MRSA LAC	64	8/3.13	0.25	16
MRSA HI168	2	0.125/12.5	0.19	16
MSSA	0.125	0.063/12.5	0.63	2
Nafcillin				
MRSA USA100	128	2/12.5	0.14	64
MRSA HI168	1	0.5/12.5	0.63	2

As most *S. aureus* infections are skin and soft tissue infections,²⁶ we tested ASU014, the negative control synbody ASU042, and a vehicle control for *in vivo* activity in an air pouch model of skin infection.^{27,28} In this study, mice ($n = 8$ per group) were infected by air-pouch injection of 10^7 CFU/mL of USA300-LAC coincident with vehicle control, 100 μM ASU014, or 100 μM ASU042. The following day, mice were weighed and bacterial counts enumerated in the pouch and the kidneys. Treatment with ASU014 significantly reduced bacterial burden at the site of infection as well as dissemination to the kidneys compared to vehicle or synbody control (Figure 3A,B). Consistent with reduced bacterial burden, ASU014

Figure 3. Activity against MRSA in a murine air-pouch skin infection model. Bacterial counts (A) in the pouch or (B) disseminated to the kidneys of mice 24 h after inoculation with 2.5×10^7 CFU of USA300-LAC and treated with vehicle control (VC), ASU042 control, or 100 μM ASU014. (C) Percent weight loss of infected mice. Data shown are mean $\pm$ SEM; $n = 8$ mice per group. Mann–Whitney test for nonparametrics; * $p < 0.05$, *** $p < 0.001$.

treated mice lost less weight, an indicator of overall morbidity, compared to controls (Figure 3C). Although our *in vitro* findings were promising, many AMPs, due to their cationic charge, nonspecifically interact with competing proteins and lose activity in the presence of serum proteins and physiological salt concentrations.¹² Therefore, these results demonstrate that ASU014 functions as a monotherapy in the context of an *in vivo* infection model with competing serum proteins, interstitial fluid, and tissue.

We next evaluated if the *in vitro* synergy observed with Oxa would translate to *in vivo* function in the air pouch model. As before, BALB/c mice were challenged with USA300-LAC plus vehicle control, 64 $\mu\text{g/mL}$ Oxa, 12.5 μM ASU014, or the combination of 64 $\mu\text{g/mL}$ Oxa and 12.5 μM ASU014. The following day, bacteria were collected from the pouch lavage or

the kidneys, plated, and enumerated. In the pouch, only the combination of ASU014 and Oxa significantly reduced bacterial burden compared to vehicle control ($p < 0.05$) (Figure 4A).

Figure 4. Adjunctive benefit with oxacillin against MRSA in an *in vivo* pouch model. Mice were treated with vehicle control, 12.5 μ M ASU014, 64 μ g/mL Oxa, or 12.5 μ M ASU014 + 64 μ g/mL Oxa coincident to infection with 7.5×10^7 CFU of USA300-LAC. Bacterial burden (A) in the pouch or (B) disseminated to the kidneys 24 h postinfection, and (C) percent weight loss in infected mice. Data are mean $\pm$ SEM; $n = 8$ –12 mice per group. Mann–Whitney test for nonparametrics; * $p < 0.05$, ** $p < 0.01$.

Dissemination to the kidneys did not significantly differ between the groups, despite a slight reduction in bacterial burden with combination treatment (Figure 4B). However, consistent with reduced bacterial burden at the site of infection, only combination treated mice lost less weight versus vehicle control ($p < 0.01$) (Figure 4C). These data demonstrate that sub-MIC concentrations of ASU014 can be used to improve the activity of Oxa against MRSA *in vivo*.

The results presented here demonstrate that ASU014 has several notable attributes for future development. First, ASU014 functions in a biological matrix in the presence of competing proteins, proteases, salts, and epithelial cells, despite the fact that the thiol–succinimide conjugation site is sensitive to exchange reactions, as demonstrated by our group and others.^{22,29} This should limit the function of ASU014 and would be an obvious target for optimization. While ASU014 is a mixture of structural isomers, we have not seen evidence of the peptide attachment site affecting the activity of previous antibacterial synbodies and do not anticipate it having a large effect on activity. However, future optimization of ASU014 would involve site selective peptide conjugation in order to produce a single product. Second, the modular nature of the bifunctional scaffold used in the study lends itself to easy modulation of intrapeptide orientation providing another method to optimize anti-*Staphylococci* activity. Finally, while the air pouch model used in this study is not necessarily predictive of activity upon systemic delivery of ASU014, it does demonstrate function in an *in vivo* model. While ASU014 has mild single agent activity, it synergizes with β -lactams at 0.25 to 0.125 MIC. This suggests that this molecule could be used with narrow spectrum β -lactams to offer selective treatment of MRSA infections in much the same way that daptomycin/oxacillin combinations are under exploration as combination treatments for MRSA infected patients. Overall, this data demonstrates that ASU014 is a candidate for future medicinal chemistry optimization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00200.

Protocols for peptide array assay; synbody conjugation and characterization; growth inhibition and checkerboard assays; *in vivo* protocols; table of peptide sequences; negative control synbody description; bacteria strain information (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1-480-727-4264. E-mail: chris.diehnelt@asu.edu.

ORCID

Chris W. Diehnelt: 0000-0003-4565-9338

Author Contributions

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Notes

The authors declare the following competing financial interest(s): C.W.D., S.A.J., and P.R.H. have a patent application filed on synbody compositions described in this manuscript.

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