Efficacious Analogs of the Lantibiotic Mutacin 1140 against a Systemic Methicillin-Resistant *Staphylococcus aureus* Infection

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ABSTRACT Mutacin 1140, a member of the epidermin family of type AI lantibiotics, has a broad spectrum of activity against Gram-positive bacteria. It blocks cell wall synthesis by binding to lipid II. Although it has rapid bactericidal effects and potent activity against Gram-positive pathogens, its rapid clearance and short half-life *in vivo* limit its development in the clinic. In this study, we evaluated the effect of charged and dehydrated residues on the pharmacokinetics of mutacin 1140. The dehydrated residues were determined to contribute to the stability of mutacin 1140, while alanine substitutions for the lysine or arginine residues improved the pharmacological properties of the antibiotic. Analogs K2A and R13A had significantly lower clearances, leading to higher plasma concentrations over time. They also had improved bioactivities against several pathogenic bacteria. In a murine systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infection model, a 10-mg/kg single intravenous bolus injection of the K2A and R13A analogs (1:1 ratio) protected 100% of the infected mice, while a 2.5-mg/kg dose resulted in 50% survival. The 10-mg/kg treatment group had a significant reduction in bacteria load in the livers and kidneys compared to that in the vehicle control group. The study provides lead compounds for the future development of antibiotics used to treat systemic Gram-positive infections.

KEYWORDS MRSA, lantibiotics, mutacin

Lantibiotics are lanthionine-containing, ribosomally synthesized posttranslationally modified peptides (RiPPs) with antimicrobial activities (1). A serine or threonine residue is posttranslationally dehydrated into a 2,3-didehydroalanine (Dha) or 2,3-didehydrobutyrine (Dhb) residue, respectively. A lanthionine (Lan) or methylanthionine (MeLan) ring is formed between a cysteine residue and a Dha or Dhb residue, respectively, through a thioether linkage (2). Type AI lantibiotics, including the nisin and epidermin families, have been extensively studied and have a unique mechanism of action against Gram-positive bacteria compared to that of other antibiotics currently in use in the clinic. They share a conserved lipid II binding motif, which consists of the N-terminal rings A and B. The targets of the motif are the pyrophosphate, peptidoglycan MurNAc, and the first isoprene of cell wall precursor lipid II (3, 4).

Nisin and mutacin 1140 have potent activities (nanomolar or submicromolar activity) against well-known Gram-positive pathogens, including *Staphylococcus aureus* and *Streptococcus pneumoniae*. Nisin has been used as a food preservative for more than 50 years without inducing significant resistance (5). Sequential subculturing of *S. aureus* ATCC 25923 in subinhibitory concentrations of mutacin 1140 for 21 days only led to a 2-fold increase in the MIC (6). However, nisin and mutacin 1140 have poor pharmacokinetics and are susceptible to proteolytic degradation. Nisin can be inactivated with chymotrypsin, trypsin, and thermolysin digestion (7), and mutacin 1140 can be inactivated by trypsin digestion (6). After intravenous administration, nisin was found to have

Received 31 July 2018 Returned for
modification 15 September 2018 Accepted
21 September 2018

Accepted manuscript posted online 1
October 2018

Citation Geng M, Ravichandran A, Escano J,
Smith L. 2018. Efficacious analogs of the
lantibiotic mutacin 1140 against a systemic
methicillin-resistant *Staphylococcus aureus*
infection. Antimicrob Agents Chemother
62:e01626-18. <https://doi.org/10.1128/AAC.01626-18>.

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FIG 1 A representative covalent structure of mutacin 1140 and analogs used in this study. The four lanthionine rings are labeled A, B, C, and D. Protease-susceptible residues Lys and Arg are shaded, while dehydrated residues Dha and Dhb are shown in *italics*. Amino acid abbreviations in bold font represent analogs with higher peak plasma concentrations, those shaded in gray represent analogs with decreased peak plasma concentrations, while the rest of the analogs had peak plasma concentrations similar to that of the native mutacin 1140. The lipid II binding domain consists of the rings A and B, while the lateral assembly domain consists of the hinge region (residues 12 to 15) and rings C and D.

a half-life of 0.9 h in a mouse model (8), while mutacin 1140 demonstrated a half-life of 1.6 h in a rat model (9). While the reason for the short half-life of nisin remains unknown, the major reason for the short half-life of mutacin 1140 may be attributed to the relatively high rate of clearance (9). Multiple physiological factors may control the rate of clearance, and we hypothesize that the Lys and Arg residues may contribute to enzymatic degradation *in vivo* (Fig. 1), while the dehydrated residues may be susceptible to nucleophiles under physiological conditions (10).

The pharmacology of an antibiotic, i.e., the pharmacokinetics (PK) and pharmacodynamics (PD), provides the best predictive measures for efficacy in treating an infection (11). Although the half-life of a drug is one of the most important PK parameters, it is also one of the most variable PK predictions. The half-life of the same drug can vary up to 12-fold due to the sensitivity of the analytical technique used or the terminal phase estimates used for its estimation (12). Given that time-dependent and concentration-dependent variables are attributed to the inhibitory activity of antibiotics, a different approach has been proposed for evaluating the pharmacokinetic data of antibiotics (13). Maintaining the serum concentration above the MIC (time above MIC [$T > \text{MIC}$]) is one of the most important factors for the effectiveness of an antibiotic that is concentration independent in the kill rate; in other words, increasing the antibiotic concentration above the MIC has little effect on changing the rate of inhibition (14). The effectiveness of these antibiotics is generally improved with a short dosing interval or with the use of a continuous (15) or prolonged infusion that keeps the serum concentration above the MIC (16). For antibiotics that have concentration-dependent kill rates, in other words, the rate of inhibition is increased with increasing concentrations of the antibiotic above the MIC, the area under the concentration-time curve (AUC) relative to the MIC (AUC/MIC) against the bacterial pathogen is the most important factor linked to their effectiveness for treating an infection (17, 18).

Despite the short half-life of mutacin 1140 in blood, analogs of mutacin 1140 were demonstrated to have increased gastric stability and were effective in treating a *Clostridium difficile* infection in hamsters (19). Furthermore, several mutagenesis studies on mutacin 1140 have focused on the permissiveness of core peptide modifications for improving its stability against proteases (6, 20, 21). However, it is not clear if analogs with higher *in vitro* protease stability or the removal of potentially reactive dehydrated

FIG 2 Plasma concentration-time profiles of native mutacin 1140 and the K2A, S5G, R13A, T14A, K2A:R13A, S5A:T14G, and S5G:R13A:T14A analogs. The intravenous dose for all analogs was 2.5 mg/kg, except for the K2A and K2A:R13A analogs. Due to their lower solubility in saline, these two analogs were administered at 1.57 mg/kg and 2.08 mg/kg, respectively. Blood was sampled at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h postinjection. Native mutacin 1140 or mutacin 1140 analogs were not detected after 2 h; thus, the later time points are not shown in the figure. ●, K2A analog; ■, R13A analog; ▲, K2A:R13A analog; ◆, native mutacin 1140; ●, S5G analog; ◆, T14A analog; ■, S5A:T14G analog; and ▲, S5G:R13A:T14A analog. Some error bars are not visible, since they are smaller than the symbol used.

residues will lead to improved pharmacokinetics *in vivo*. To date, there is no report on the *in vivo* efficacy of mutacin 1140 or an analog of mutacin 1140 in a systemic infection model. In this study, analogs K2A and R13A were identified to have improved pharmacokinetic profiles, and a combination of the two analogs was shown to be efficacious in treating systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infections in mice.

RESULTS

Dehydrated residues contribute to the stability of mutacin 1140 *in vivo*. The mouse model was used to study the effect of dehydrated residues Dha5 and Dhb14 on *in vivo* stability (Fig. 2). The native mutacin 1140 and the analogs S5G, T14A, S5A:T14G, and S5G:R13A:T14A were administered intravenously at a dose of 2.5 mg/kg. Compared to that of the native mutacin 1140, the analogs with one or more amino acid substitutions in the Dha5 and Dhb14 positions had lower peak plasma concentrations and had lower plasma concentrations of the drugs at all time points. The analogs without any dehydrated residues, namely, S5A:T14G and S5G:R13A:T14A, had the lowest peak plasma concentrations, which were more than 5-fold lower than for the native mutacin 1140. The analogs with single substitutions, namely, S5G and T14A, performed better than the S5A:T14G and S5G:R13A:T14A analogs. However, the single substitutions still performed worse than the native compound. The T14A analog had a more than 3-fold lower peak plasma concentration, while the S5G analog only had a moderate reduction in peak plasma concentration compared to that of the native mutacin 1140. The reason for the reduced plasma concentrations remains unclear. It is possible that the two dehydrated residue positions contribute to an important structural conformation of mutacin 1140 that is vital to maintain its *in vivo* stability.

Substitution of one of the two protease-susceptible residues improves the mean residence time of mutacin 1140 *in vivo*. Native mutacin 1140 and the analog R13A were administered intravenously at a dose of 2.5 mg/kg. Due to the reduced solubility of the K2A and K2A:R13A analogs in saline, they were administered at 1.6 mg/kg and 2.1 mg/kg, respectively. The analogs with one amino acid substitution at the protease-susceptible lysine or arginine positions had higher plasma concentrations than the native mutacin 1140 during all time points (Fig. 2). The K2A analog had the highest peak plasma concentration among all of the analogs tested, which was approximately 2-fold higher than the native compound at 15 min postinjection (mpi). At 15 mpi, the R13A analog had a higher plasma concentration than the native mutacin 1140. Additionally, it had the highest plasma concentration at 30 mpi and 60 mpi

FIG 3 *In vivo* and *in vitro* stability of native mutacin 1140 and the K2A and R13A analogs. (A) Plasma concentration-time profiles in the 10-mg/kg dosing study. Blood was sampled at 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 8 h postinjection. Native mutacin 1140 or the analogs could not be detected at 8 h; thus, the 8-h time point is not shown in the figure. The area under the curve ratios of each analog to that of native mutacin 1140 are shown. *, $P < 0.05$ by Student's *t* test. (B) Expansion of the plasma concentration-time profiles from 30 min to 90 min postinjection. (C) Serum concentration-time profiles of mutacin 1140 analogs. ●, K2A analog; ■, R13A analog; and ◆, native mutacin 1140. The K2A and R13A analogs are shown as solid lines, while native mutacin 1140 is shown as a dashed line. The initial concentration of native mutacin 1140 and the K2A and R13A analogs in the mouse serum was 1,000 ng/ml. The samples were incubated at 37°C for 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, and 6 h. The experiments were performed in duplicates. Some error bars are not visible, since they are smaller than the symbol used.

among all the tested compounds. Counterintuitively, the K2A:R13A analog, which lacks both charged residues, had a lower peak plasma concentration than the analogs K2A and R13A. Furthermore, it was cleared by 60 mpi. In a previous study, we demonstrated that the K2A:R13A analog was more resistant to trypsin digestion than the K2A and R13A analogs (6). It is possible that the combination of substitutions in the N terminus and hinge region plays a role in altering the overall predominant structural conformation and thus the stability of mutacin 1140.

The K2A and R13A analogs, which had improved pharmacokinetic parameters, were administered intravenously to mice at a dose of 10 mg/kg for further comparison to the native compound. The three compounds were dissolved at 2 mg/ml in 5% dimethyl sulfoxide (DMSO) in saline to enable the higher dose, which provided additional time point measurements at 45 mpi and 90 mpi (Fig. 3). The differences of the peak plasma concentrations for the analogs compared with that of the native compound were

similar to those observed in the lower dose study. The K2A analog had 3.5-fold and 2.9-fold higher peak plasma concentrations than native mutacin 1140 and the R13A analog, respectively. At 30 mpi, it had 2.8-fold and 2.2-fold higher plasma concentrations than native mutacin 1140 and the R13A analog, respectively. At 45 mpi, it had a 3.2-fold higher plasma concentration than the native compound but only a 1.7-fold higher concentration than the R13A analog. At 60 mpi, the R13A analog had the highest plasma concentration, which was 1.6-fold higher than that of the K2A analog and 5.7-fold higher than that of the native compound. The R13A analog appears to be cleared from the blood much slower than the native mutacin 1140 and K2A analog (Fig. 3B). The R13A analog remained detectable in blood for 6 h, while the K2A analog was not detected after 4 h and the native mutacin 1140 was not detected after 2 h. The AUCs of the K2A and R13A analogs were 3.35- and 1.62-fold higher, respectively, than the AUC of native mutacin 1140 ($P < 0.05$) (Fig. 3A). The lower rate of clearing of the R13A analog in plasma may be attributed to its higher resistance to a trypsin-like protease in blood or to other enzymatic degradation. However, the 4-fold slower clearance of the K2A analog suggests that something else may be involved with the improved stability, due to the fact that the K2A analog does not show any resistance to trypsin digestion (6).

An *in vitro* pharmacokinetics study was performed to evaluate if the improved *in vivo* pharmacokinetics can be partly attributed to the improved stability in mouse serum (Fig. 3C). After a 15-min incubation in mouse serum at 37°C, the concentration of native mutacin 1140 dropped 37%, whereas the concentrations of the K2A and R13A analogs did not decrease. After a 1-h exposure, the remaining native mutacin 1140 and the R13A analog concentrations were less than 50%, while the remaining concentrations of the K2A analog exceeded 80% of the initial concentration. After 2 h of exposure, there was approximately 20% of the initial concentrations for the native compound and the R13A analog, while the amount of the K2A analog remaining was >40% of the initial concentration. The native compound was not detectable after 4 h, while both the K2A and R13A analogs were detectable for 6 h. When an exponential model was used, the *in vitro* half-lives of the native compound and the K2A and R13A analogs were calculated to be 0.9, 1.5, and 1.1 h, respectively. The *in vitro* stability data match the *in vivo* data, suggesting that enzymatic degradation of mutacin 1140 in serum presumably plays a major role in the rapid clearance observed *in vivo* and that the removal of Lys or Arg residues contributes to the stability of the mutacin 1140 analogs.

Mutacin 1140 analogs exhibit a rapid killing effect. To better predict the efficacy of mutacin 1140 analogs *in vivo*, a time-kill study was performed using native mutacin 1140 and the K2A and R13A analogs against two pathogenic strains of *S. aureus* (ATCC 25923 and ATCC 33591) (Fig. 4). The *S. aureus* ATCC 25923 strain was chosen for kill kinetics because it was the least sensitive pathogenic bacterial strain used in our previously published study (6), while the methicillin-resistant ATCC 33591 strain has been widely used in efficacy studies (22–24). The MICs of mutacin 1140 analogs and of vancomycin against *S. aureus* strains ATCC 25923 and ATCC 33591 are listed in Table 1. The methicillin-resistant ATCC 33591 strain was 2-fold and 8-fold more sensitive to the K2A and R13A analogs, respectively, than to the native mutacin 1140. Different concentrations (0.25×, 0.5×, 1×, and 2× MIC) of each analog were tested in time-kill studies.

All three compounds exhibited a rapid bactericidal effect against the ATCC 25923 strain. At 1× and 2× MICs, there was more than a 3-log reduction in cell density at the 15-min time point, and there was no regrowth by the 24-h time point. Therefore, these data are not shown in Fig. 4A. The R13A analog at 0.25× MIC had a moderately better bactericidal effect than the native compound. There were 200-fold and a 400-fold reductions in viable cell counts for native mutacin 1140 and the R13A analog, respectively, within 15 min. The inhibition of growth lasted for approximately 2 h, and the effect of the 0.25× MIC dose of the R13A analog was still clear by 8 h. The viable cell

FIG 4 Kill kinetics for native mutacin 1140 and the K2A and R13A analogs against two *S. aureus* strains. (A) Kill kinetics against *S. aureus* ATCC 25923. The 0.25 $\times$ MIC treatments are shown in solid lines, while the 0.5 $\times$ MIC treatments are shown as dashed lines. For all the 1 $\times$ MIC and 2 $\times$ MIC treatments, viable cell counts were below the detection limit for all time points tested and thus are not shown in the figure. (B) Kill kinetics against *S. aureus* ATCC 33591 at 1 $\times$ and 2 $\times$ MIC. The 1 $\times$ MIC treatments are shown as solid lines, while the 2 $\times$ MIC treatments are shown as dashed lines. (C) Kill kinetics against *S. aureus* ATCC 33591 at 0.25 $\times$ and 0.5 $\times$ MIC. The 0.25 $\times$ MIC treatments are shown as solid lines, while the 0.5 $\times$ MIC treatments are shown as dashed lines. ■, no drug control; ▲, native mutacin 1140; ●, K2A analog; and ◆, R13A analog. The lowest detection limit of viable cell counts is 100 CFU/ml. The experiments were performed at least in duplicates. Some error bars are not visible, since they are smaller than the symbols used.

counts were still 2-log lower than for the no drug control. The K2A analog had the best killing effect during the first 2 h at 0.25 $\times$ MIC. The viable cell counts at the 2-h time point were approximately 5-fold lower than those for both the native mutacin 1140 and the R13A analog, and at 18 h, the effect was still pronounced, with a 10-fold decrease in viable cell counts compared to those for the native mutacin 1140 and the R13A analog. The data show that the K2A analog had a prolonged inhibition effect at its 0.25 $\times$ MIC value. At the 0.5 $\times$ MIC value, the K2A and R13A analogs had a slightly better inhibition effect than the native compound. The viable cells counts were below the limit of quantification within 2 h of drug exposure. The regrowth of the culture began after 4 h, and the culture reached the cell density of the no drug control by 24 h. The K2A analog-treated culture still had approximately 1-log fewer cells than that treated with the native mutacin 1140 at 18 h.

TABLE 1 MICs of native mutacin 1140 and K2A and R13A analogs against two *S. aureus* and one *S. pneumoniae* strain in broth and in 50% mouse serum^a

Mutacin 1140 type	MIC ($\mu\text{g/ml}$)					
	<i>S. aureus</i> ATCC 33591		<i>S. aureus</i> ATCC 25923		<i>S. pneumoniae</i> AI7	
	In broth	In 50% serum	In broth	In 50% serum	In broth	In 50% serum
Wild type	0.5	2	8	64	0.25	1
K2A analog	0.25	1	32	>64	0.125	2
R13A analog	0.0625	0.25	16	64	0.25	1
Vancomycin	2	4	ND ^b	ND	ND	ND

^aPart of the data in the table has been previously reported (6). Vancomycin serves as a comparison for the efficacy study and thus was only evaluated against the MRSA ATCC 33591 strain.

^bND, not determined.

Compared to that against the ATCC 25923 strain, the native mutacin 1140 and the K2A and R13A analogs exhibited a slower killing effect against the ATCC 33591 strain. At $1 \times \text{MIC}$ (Fig. 4B), there was a >2 -log reduction in the number of viable cells after a 1-h exposure with the K2A and R13A analogs, while $1 \times \text{MIC}$ of the native mutacin 1140 produced a >3 -log reduction in viable cells. The viable cell numbers fell below the detection limit after 2 h of exposure with the K2A analog, after 4 h of exposure with the R13A analog, and after 90 min of exposure with the native compound. At $2 \times \text{MIC}$ exposure for 15 min (Fig. 4B), there were 2-log reductions in the numbers of viable cells for all three compounds. Sub-MIC exposures (Fig. 4C) with native mutacin 1140 and the K2A and R13A analogs inhibited bacterial growth for at least 4 h. Compared to that with $0.25 \times \text{MIC}$ exposure, the $0.5 \times \text{MIC}$ exposure resulted in 1-log fewer viable cells at 8 h. However, there was no significant difference between the cell densities at 18 h.

In vivo efficacy study using lead mutacin 1140 analogs. A previous report showed that more than 90% of mutacin 1140 was bound to human serum proteins. Serum binding of mutacin 1140 resulted in a reduction of the *in vitro* inhibitory activity against *S. pneumoniae*, but the presence of serum did result in an increase in activity against *S. aureus* (25). Our *in vitro* stability assay showed that mutacin 1140 analogs were rapidly degraded in mouse serum at 37°C . The MICs of the K2A and R13A analogs and the native compound were tested against two strains of *S. aureus* and one strain of *S. pneumoniae* in the presence of 50% mouse serum. The presence of the serum resulted in a 4 to 16-fold reduction in inhibitory activity. However, the K2A and R13A analogs had 2-fold and 8-fold increases in activity, respectively, against the MRSA strain ATCC 33591 compared with that of the native compound (Table 1). According to the parameters derived from a noncompartmental analysis (NCA) (Table 2), the K2A analog had the lowest total clearance (CL) and the highest AUC, while the R13A analog had the highest volumes of distribution at steady state (V_{ss}) and the longest mean residence time (MRT) and half-life ($t_{1/2}$). With respect to the native compound, the data suggest that the K2A analog has a superior AUC/MIC property, while the R13A analog has a superior $T > \text{MIC}$ property. Given their different PK profiles, we decided to test a combination of the two analogs (1:1 ratio) in an *in vivo* systemic infection model. Another reason for combining the two analogs was that the two analogs have various activities and specificities against different Gram-positive bacteria (6); thus, the use of a combination of the two is more advantageous when treating unknown Gram-positive infections. This is due to the fact that multiple pathogenic strains may be present

TABLE 2 Noncompartmental analysis of native mutacin 1140 and the K2A and R13A analogs following a 10-mg/kg i.v. administration^a

Mutacin type	AUC _{0-t} ($\mu\text{g}\cdot\text{h/ml}$)	CL (liter/kg/h)	$t_{1/2}$ (h)	V_{ss} (liter/kg)	C_{max} ($\mu\text{g/ml}$)	MRT (h)
Wild type	3.13 ± 0.32	3.22 ± 0.31	0.32 ± 0.11	0.54 ± 0.09	3.78 ± 0.34	0.17 ± 0.02
K2A analog	12.38 ± 2.66	0.85 ± 0.16	0.72 ± 0.15	0.13 ± 0.04	13.20 ± 1.79	0.15 ± 0.02
R13A analog	4.05 ± 0.19	2.46 ± 0.14	2.04 ± 1.03	0.98 ± 0.25	4.61 ± 0.19	0.41 ± 0.13

^aPlasma concentration-time data performed by PKSolver 2.0 (38). Parameters include the area under the concentration-time curve (AUC_{0-t}), total clearance (CL), half-life ($t_{1/2}$), volume of distribution at steady state (V_{ss}), maximum plasma concentration (C_{max}), and mean residence time (MRT). All numbers were rounded up to two decimal places.

FIG 5 Efficacy study using the K2A and R13A analogs (1:1) in a murine MRSA systemic infection model. (A) Survival curves from the day of infection (day 0) to day 5. (B) Bacterial loads (log CFU/g) in kidneys. (C) Bacterial loads (log CFU/g) in livers. The symbols indicate log CFU/g recovered from individual organs, while the horizontal lines indicate the averages from each group. The symbols on the x axes indicate that the number of colonies recovered was below the detection limit. *, $P < 0.05$ versus the vehicle control by Student's t test. As the 2.5-mg/kg treatment group contains fewer samples than the vehicle control and the 10-mg/kg treatment groups, statistical analyses were not performed. ●, vehicle control group ($n = 6$); ▲, 10-mg/kg treatment group ($n = 6$); and ■, 2.5-mg/kg treatment group ($n = 4$).

during an infection or that the antibiotic sensitivities of the infective strains are unknown at the time treatment is needed.

A murine systemic MRSA infection model was developed to investigate the *in vivo* efficacy of the two lead mutacin 1140 analogs. Equal numbers of male and female mice were used in each group. A lethal infection was achieved via an intraperitoneal (i.p.) injection of approximately 1.5×10^8 CFU of *S. aureus* ATCC 33591 per mouse. Intravenous (i.v.) injections in the tail veins were performed 1 h postinfection. One group of mice ($n = 6$) received vehicle, one group ($n = 6$) received the antibiotic at 10 mg/kg (5 mg/kg each analog), while the last group ($n = 4$) received the antibiotic at 2.5 mg/kg (1.25 mg/kg each analog) (Fig. 5). We monitored the mice for 5 days. The group that received the vehicle had 33.3% mortality within 24 h and 100% mortality by 48 h. All mice survived following i.v. administration of 10 mg/kg of the K2A and R13A analogs, while only half of the mice in the 2.5-mg/kg treatment group survived.

Bacterial loads in the kidneys and livers (Fig. 5B and C) were determined at the time the mice were sacrificed. The 10-mg/kg treatment group had a >1 -log reduction in bacterial load in the kidneys and an approximately 2-log reduction in the livers compared to those in the vehicle control. Both of the reductions were statistically significant ($P < 0.05$). The bacterial loads of the 2-mg/kg treatment group varied significantly given that half of the mice died from the infection. One of the 2.5-mg/kg treated mice that survived the 5-day study had bacterial loads below the quantification limit in the kidney and liver. We were not able to determine the statistical significance of the difference in bacterial loads between the control and the 2.5-mg/kg treatment groups or between the 10-mg/kg and the 2.5-mg/kg treatment groups due to the fact that we only had four samples from the 2.5-mg/kg treatment group. Further investigation is needed, but the data suggest that a single 2.5-mg/kg dose might be the 50% effective dose (ED_{50}).

DISCUSSION

The dehydrated and protease-susceptible residues within mutacin 1140 were evaluated in this study to determine whether they contribute to its *in vivo* stability. We generated plasma concentration-time profiles of mutacin 1140 and its analogs with fewer dehydrated and/or protease-susceptible residues following intravenous administration in a mouse model. We also tested the *in vivo* efficacy of the two analogs with improved pharmacokinetics profiles. Our findings revealed that (i) dehydrated residues Dha5 and Dhb14 are important for the *in vivo* stability of mutacin 1140, (ii) the K2A and R13A analogs had higher peak plasma concentrations, higher AUCs, and slower clearance than the native mutacin 1140, while the combined analog K2A:R13A was cleared from plasma faster than the native mutacin 1140, and (iii) the K2A and R13A analogs exhibited rapid bactericidal effects against two *S. aureus* strains, such that a single intravenous administration of the lead analogs (K2A and R13A; 1:1 ratio) at 10 mg/kg is efficacious for treating a systemic MRSA infection in mice.

An elimination terminal half-life of 1.6 h was previously reported for the native mutacin 1140 in rats after an intravenous injection (9), which is discouraging for its continued development for the treatment of a systemic infection. Since these reports, extensive mutagenesis work on the core peptide has been done to generate structural variants of mutacin 1140 with improved bioactivity and proteolytic stability (6, 19, 20). In a previous study, mutacin 1140 analogs with fewer dehydrated residues and/or protease-susceptible residues were generated (6). It was shown that the K2A:R13A analog was completely resistant to trypsin digestion, while the R13A analog was partially resistant to trypsin digestion. The K2A analog remained susceptible and was completely inactivated after exposure to trypsin. However, none of these analogs had been tested *in vivo*. After an intravenous administration at a dose of 2.5 mg/kg or less, all analogs had a rapid drop in plasma concentration from 15 mpi to 30 mpi and were barely detectable by 60 mpi. The present study does show that the dehydrated residues as well as a combination of N terminus and hinge region residues are important for the *in vivo* stability of mutacin 1140. There was a >10-fold difference between the K2A and S5G:R13A:T14A analogs in peak plasma concentrations. We suggested in a previous study that Dha5 and Dhb14 residues may contribute to an important structural conformation of mutacin 1140 (6), which could improve the *in vivo* stability. Nonetheless, the *in vivo* stability of mutacin 1140 in blood is also attributed to other physical attributes of the peptide. The K2A and R13A analogs also had improved *in vitro* stability in mouse serum, which may also explain the decreased clearance of these analogs observed *in vivo*. A better understanding of their increased stability in blood may further promote their application for treating a Gram-positive infection.

S. aureus ATCC 33591 was used for the infections in this study, since it is methicillin resistant and has been widely used in systemic infection studies (22–24). Our *in vivo* efficacy experiment supported the hypothesis that the combination of the K2A and R13A analogs is efficacious in the systemic infection model. A single intravenous dose at 10 mg/kg protected 100% of the mice for at least 5 days. To date, there are very few studies aimed at evaluating the *in vivo* efficacy of lantibiotics in systemic infection models. The first investigation on the efficacy of a mutacin B-Ny266 showed that intraperitoneal infection with a methicillin-susceptible *S. aureus* strain in mice can be protected by simultaneous intraperitoneal administration of mutacin B-Ny266 (26). However, the drug was administered at the same time through the same route of administration as the bacterial infection, which opens the question of whether mutacin B-Ny266 would be efficacious for treating infections that have already developed and whether it could be efficacious via intravenous administration. Microbisporicin has potent activity against murine septicemia caused by *S. aureus*, and it protected 50% of the mice at a 2.1-mg/kg dose via both intravenous and subcutaneous administrations (27). However, the bacterial load used for infection was only $\sim 10^6$ CFU per mouse, and no vehicle control was included in the study, making it hard to determine the efficacy of the antibiotic. A more recent study (28) demonstrated that microbisporicin had an

ED₅₀ of 14.2 mg/kg via i.v. administration 10 to 15 min after administering the *S. aureus* infection in a neutropenic mice model.

Although a direct comparison of the *in vivo* efficacies between different studies is not possible, the combination of the K2A and R13A analogs is promising for treating a systemic MRSA infection. The bacterial load administered in this study was approximately 5-fold higher than the load administered in other studies. The infection was acute, and all mice treated with the vehicle died within 2 days postinfection. The antibiotic treatment was also given 1 h postinfection, which is longer than in the previously described lantibiotics efficacy studies. Most of studies on the efficacy of nonlantibiotic antibiotics in treating systemic infections required multiple administrations. Lysostaphin was administered at 5 mg/kg once a day for 3 days to clear the infection in the systemic *S. aureus* infection model (29). Telavancin was administered 4 and 16 h postinfection at 40 mg/kg to protect 100% of the mice (24). Given the rapid clearance of the K2A and R13A analogs in blood, a multidosing strategy might promote the efficacy, which will be investigated in the future. Another aspect to further improve the antibiotic is the formulation of the lead K2A and R13A analogs. While the studies on lantibiotics formulations are limited, there are some formulations of nisin with improved features. For example, one study generated an antimicrobial nanofiber wood dressing by electrospinning nisin into poly(ethylene oxide) and poly(D,L-lactide) blend nanofibers (30). Whether mutacin 1140 analogs can be formulated by the addition of excipients (31) to improve their solubility or formulated into particles that can be slowly released into blood to increase the $T > \text{MIC}$ will be investigated in the future.

The fast *in vivo* clearance combined with the rapid *in vitro* bactericidal activity is a property of mutacin 1140 that is unique to the antibiotics currently used in the clinic, making it difficult to make a direct comparison of its inhibitory activity with those of other antibiotics. The AUC/MIC for 24 h is the nearest predictor of the therapeutic effect of aminoglycosides and quinolones (17, 18). The killing rates of the two classes of antibiotics are concentration dependent over a broad concentration range, ranging from $0.25 \times \text{MIC}$ to $24 \times \text{MIC}$. At the highest concentrations ($24 \times \text{MIC}$) tested, 2 h is required for tobramycin and ciprofloxacin to kill all bacteria (11). Vancomycin has a relatively long half-life of 6 to 12 h in humans after intravenous administration with a standard infusion time of 1 h (32), but it also has a slow killing effect against susceptible bacteria. Even at $10 \times \text{MIC}$, the data show that it took 10 h for *S. aureus* viable cell counts to drop 1,000-fold (33). Although the current study demonstrated the *in vivo* efficacy of the K2A and R13A analogs, much more work is needed to develop a model to predict its efficacy against different systemic infections.

The emergence of methicillin-resistant *S. aureus* (MRSA) and drug-resistant *S. pneumoniae* have become a severe problem in the clinic (34). Vancomycin has been referred to as an “antibiotic of last sort,” while vancomycin-resistant strains of *S. aureus* have been isolated (35). Thus, the development of novel antibiotics for the treatment of MRSA infections is urgent. The demonstration of the *in vivo* efficacy of the combination of the K2A and R13A analogs provides lead compounds for future development as an alternative antibiotic treatment option. Other strengths include its unique mechanism of action, rapid bactericidal effect, and low potential to develop resistance. In a previous study, it was shown that subculturing of *S. aureus* ATCC 25923 in subinhibitory concentrations of native mutacin 1140 for 21 days only led to a 2-fold increase in the MIC, while there was no increase in the MIC for the R13A analog (6).

In summary, this *in vivo* study evaluated the dehydrated and protease-susceptible residues for their potential role in the rapid clearance of mutacin 1140 from blood and developed lead compounds for future development aimed at treating systemic Gram-positive infections. The study showed that the dehydrated residues contribute to the stability of mutacin 1140, which is in contrast to that for the protease-susceptible residues lysine and arginine. The K2A and R13A analogs had improved bioactivities against several pathogenic bacteria and rapid bactericidal activity against two *S. aureus* strains, as well as *in vivo* pharmacokinetics. Furthermore, a single intravenous bolus

TABLE 3 Strains used in this study

Strain	Characteristic	Reference or source
<i>S. aureus</i> ATCC 25923		ATCC
<i>S. aureus</i> ATCC 33591	Methicillin resistant	ATCC
<i>S. pneumoniae</i> Al7		MSU ^a
<i>S. mutans</i> JH1140	Strain producing native mutacin 1140	ATCC
JH1140 K2A	Strain producing mutacin 1140 K2A	6
JH1140 S5G	Strain producing mutacin 1140 S5G	6
JH1140 R13A	Strain producing mutacin 1140 R13A	6
JH1140 T14A	Strain producing mutacin 1140 T14A	6
JH1140 K2A:R13A	Strain producing mutacin 1140 K2A:R13A	6
JH1140 S5A:T14G	Strain producing mutacin 1140 S5A:T14G	6
JH1140 S5G:R13A:T14A	Strain producing mutacin 1140 S5G:R13A:T14A	6

^aMSU, Department of Pathobiology and Population Medicine, Mississippi State University.

administration of the combination of the K2A and R13A analogs at 10 mg/kg protected 100% of the mice that were intraperitoneally infected with MRSA ATCC 33591.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. The bacterial strains used are listed in Table 3. The growth condition for each strain was previously described (6). BALB/c mouse serum (IMSBC-SER) was purchased from Innovative Research (Novi, MI). Vancomycin hydrochloride (50-489-134) was purchased from Research Products International (Mount Prospect, IL). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or VWR (Radnor, PA) and were of at least ACS grade.

Animals. Female BALB/c mice (Charles River, Wilmington, MA) were used in the pharmacokinetic study, while equal numbers of male and female BALB/c mice (Charles River, Wilmington, MA) were used in the infection study. The procedures were approved by the institutional animal care and use committee of Texas A&M under the number 2015-0028.

MIC assay. The purification and mass determination of mutacin 1140 analogs were performed as previously described (6). The MIC assay was performed as previously described (6). A serum MIC assay was developed to better predict the bactericidal effect of mutacin 1140 analogs *in vivo*. Briefly, BALB/c mouse serum was filtered through a 0.2- μ m filter (28145-477; VWR) and used for the serial dilution of mutacin 1140 analogs instead of THyex broth, after which equal volumes of bacterial culture were added to each of the wells, resulting in a 50% serum concentration.

Drug pharmacokinetics. Nine mice were separated into three groups, and blood was drawn at specified time intervals from three mice in each group. The blood samples were collected at 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 8 h postinjection. The blood was sampled from the right tail vein (15 μ l from each mouse and a total of 45 μ l for each time point from the group of 3 mice) and collected in 1.8-ml centrifuge tubes. The blood was mixed 10:1 (vol/vol) with 6 mg/ml of the anticoagulant sodium citrate and stored on ice before being centrifuged at $15,000 \times g$ for 10 min. The plasma was collected and transferred to a clean centrifuge tube before being stored at -20°C until further use.

Mutacin 1140 and the analogs were dissolved in sterilized 0.9% saline at a concentration of 0.5 mg/ml. The suspended drug samples were centrifuged at $15,000 \times g$ for 10 min to remove any insoluble material before injection. The amount of insoluble analog in the pellet was determined by high-pressure liquid chromatography (HPLC) by comparing the analog peak area to the peak area of a mutacin 1140 standard (6). According to the determination of the amount of insoluble material in the collected pellet, the concentration of the intravenous drug dose was later adjusted to account for the loss of material due to insolubility. Mutacin 1140 and analogs were administered intravenously to the left tail veins of the mice. The determined intravenous dose was 2.5 mg/kg for native mutacin 1140 and the S5G, T14A, S5A:T14G, S5G:R13A:T14A, and R13A analogs. Due to the reduced solubility of the K2A and K2A:R13A analogs, they were first administered at 1.6 mg/kg and 2.1 mg/kg, respectively. All mice were also subcutaneously administered 20 mg/kg diphenhydramine 1 h prior to mutacin 1140 or analog dosing as previously reported to avoid a possible hypersensitivity reaction (9). Native mutacin 1140 and the K2A and R13A analogs were also administered at 10 mg/kg. Mutacin 1140 and the analogs were dissolved in DMSO at a concentration of 40 mg/ml and slowly diluted 20-fold with 0.9% saline (final concentration of 2 mg/ml). The final concentration of DMSO in the saline solution for injection was 5% for each analog.

Plasma samples were thawed at room temperature prior to extraction. The extraction of mutacin 1140 and the analogs was performed as previously reported (36), with some modifications. A combination of 70% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and 30% methanol containing 0.4% formic acid was determined to be the most efficient extraction solvent. Two volumes of extraction solvent were added to the plasma samples and vortexed for 15 to 20 s. The samples were then centrifuged at $15,000 \times g$ for 10 min, after which the supernatant was transferred to a clean centrifuge tube before being freeze-dried on a SpeedVac (catalog number 7810010; Labconco). The dried samples were brought up to the initial volumes of the plasma samples in 50% ACN with 0.1%TFA containing 1 μ g/ml of the internal standard. The T14A analog served as an internal standard of native mutacin 1140

TABLE 4 Optimized parameters used to monitor ions of each analog

Mutacin 1140 type	Center mass (<i>m/z</i>)	Scan width (<i>m/z</i>)	Collision energy (V)	Internal standard
Wild type	1133.2	0.3	10	T14A
K2A analog	1104.68	0.3	10	Wild type
S5G analog	1126.7	0.3	10	Wild type
R13A analog	1090.69	0.3	10	Wild type
T14A analog	1127.085	0.3	10	Wild type
K2A:R13A analog	1062.14	0.2	15	Wild type
S5A:T14G analog	1121.21	0.3	10	T14A
S5G:R13A:T14A analog	1078.67	0.3	10	Wild type

and the S5A:T14G analog, while the native mutacin 1140 served as the internal standard for the other analogs.

An Agilent 1200 front-end chromatography system and a TSQ Quantum Access triple quadrupole mass spectrometer was used to analyze the samples. After a 10- μ l injection, the samples were separated using a 9-min water/ACN (containing 0.2% formic acid) gradient starting from 95% to 20% water on a C₁₈ column (SinoChrom ODS-BP 5 μ m, 2.1 mm by 50 mm). The mass spectrometer was operated in positive mode and operated using a protocol optimized for each analog (listed in Table 4). The initial liquid chromatography-mass spectrometry (LC-MS) parameters were adapted from a previously described method (37), with some modifications. The area of each analog and its internal standard was measured through manual integration using Xcalibur software (Thermo Fisher Scientific). The standard curves were generated for native mutacin 1140 and for each analog after the serum extraction protocol described above. The *R*² values for each standard curve exceeded 0.99.

An *in vitro* pharmacokinetics study was conducted to evaluate the correlation between *in vitro* and *in vivo* stabilities. Mouse serum was filtered through a 0.2- μ m filter and aliquoted into 1.8-ml centrifuge tubes in 100- μ l volumes. Native mutacin 1140 and the K2A and R13A analogs were added to each tube at a final concentration of 1,000 ng/ml and were incubated at 37°C. At time points of 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 4 h, and 6 h, two tubes were removed for each analog and stored immediately at –20°C. All samples were extracted and quantified as previously described.

The AUC was calculated using the trapezoidal rule, and the *T*>MIC was determined using the equation of a straight line that connects the initial time point (*T* = 0) to the time point where the mutacin 1140 or the analog concentration falls to that of the MIC. PKSolver 2.0 (38) was used to perform the noncompartmental analysis (NCA) and to calculate the parameters listed in Table 2, including the area under the concentration-time curve (AUC_{0–t}), total clearance (CL), half-life (*t*_{1/2}), volumes of distribution (*V*_{ss}), and the mean residence time (MRT) of mutacin 1140 wild-type and the K2A and R13A analogs.

Kill kinetics. The bactericidal activity of native mutacin 1140 and the K2A and R13A analogs was determined using a time-kill study against *S. aureus* ATCC 25923 and ATCC 33591. A single colony from an overnight culture of *S. aureus* was inoculated in 10 ml fresh THYex broth and allowed to grow to an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8. The culture was diluted to an OD₆₀₀ of 0.132, after which a 150-fold or 30-fold dilution in fresh broth was performed for *S. aureus* ATCC 25923 or ATCC 33591, respectively, resulting in an initial 2.5×10^5 to 6×10^5 CFU/ml cell density. Mutacin 1140 analogs were solubilized in DMSO at a concentration of 10 mg/ml. A 10-ml aliquot of the prepared inoculum was supplemented with an appropriate amount of mutacin 1140 or analog, resulting in a final concentration equivalent to 0.25 $\times$, 0.5 $\times$, 1 $\times$, and 2 $\times$ MIC. A DMSO blank was used as a no drug control. The final concentration of DMSO was 1% or 0.1% (vol/vol) for culture of the ATCC 25923 or ATCC 33591 strain, respectively. The 50-ml tubes containing 10 ml of the culture were placed in a shaking incubator at 37°C and 200 rpm (Stuart orbital incubator SI500). For each sample, 100 μ l of the culture was removed at time points of 0 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 4 h, 8 h, 18 h, and 24 h. The culture was serially diluted (10 times) in fresh medium before being spread on THYex agar plates. The plates were allowed to grow for 16 to 24 h at 37°C before the cells were counted. The CFU/ml for each time point at each drug concentration was determined using at least two independent cultures. Plates with 30 to 300 colonies were used for CFU measurements.

Murine model of systemic MRSA infection. The K2A and R13A analogs were used in combination against the MRSA strain ATCC 33591 in a mouse systemic infection model. The infection model with some modifications has been routinely used to evaluate the *in vivo* efficacy of other antibiotics (23, 39). The reported i.p. inoculum of the MRSA strain ATCC 33591 was not sufficient to induce a lethal infection. The bacterial load used to establish a lethal infection was approximately 5-fold higher than the highest dose listed in the references. Some mice were needed to establish the infection model, leading to only four mice in the 2.5-mg/kg treatment group. Stocks of the K2A and R13A analogs were prepared at concentrations of 1 and 0.25 mg/ml per analog in 5% DMSO in saline. Each mouse was first infected via an i.p. injection with 0.5 ml of a bacterial suspension containing $\sim 1.5 \times 10^8$ CFU in phosphate-buffered saline (PBS) at pH 7.4. At 1 h postinfection, one group of mice (*n* = 6) was treated with single intravenous doses of 5 mg/kg per analog (10 mg/kg in total), one group of mice (*n* = 4) was treated with single intravenous doses of 1.25 mg/kg per analog (2.5 mg/kg in total), while the last group of mice (*n* = 6) was treated with the drug vehicle (saline with 5% DMSO). The weight of each mouse was monitored three times daily during the first 2 days postinfection (day 0 to day 2), and at least twice daily from day 2 to day 5. The mice were sacrificed when they attained a 20% weight loss or on the basis behaviors indicating pain and suffering (lethargy or abnormal locomotion). All remaining mice were sacrificed on

day five. Once sacrificed, the livers and kidneys were removed, weighed, and homogenized (47732-446; VWR) in PBS at pH 7.4 in a total volume of 1 ml. A 10-fold serial dilution in PBS was performed and plated on THyex agar plates for colony enumeration. Plates were incubated at 37°C for 18 to 24 h, and colony counts (~30 to 300 per plate) were performed and converted to CFU/g of tissue.

ACKNOWLEDGMENTS

We thank Bindu Nanduri, Department of Basic Sciences, Mississippi State University, for use of the clinical *S. pneumoniae* isolate AI7.

These experiments were in part supported by NIH grants 1R41AI131792-01 and 1R41AI122441-01A1.

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