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Pharmacodynamic activity of the lantibiotic MU1140

Oliver G. Ghobrial^{a,b}, Hartmut Derendorf^b, and Jeffery D. Hillman^{a,*}

^a *Oragenics Inc., 13700 Progress Blvd., Alachua, FL 32615, USA*

^b *Department of Pharmaceutics, University of Florida, Gainesville, FL 32611, USA*

Abstract

This study evaluated the pharmacodynamics of the lantibiotic MU1140 and the ability of selected organisms to develop resistance to this antibiotic. MU1140 demonstrated activity against all Gram-positive organisms tested, including oxacillin- and vancomycin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (VREF). No activity was observed against Gram-negative bacteria or yeast. Time–kill studies revealed that MU1140 was rapidly bactericidal against *Streptococcus pneumoniae* and multidrug-resistant *S. aureus*, whilst it was bacteriostatic against VREF. In vitro resistance development to MU1140, tested by sequential subculturing in subinhibitory concentrations of MU1140, revealed a stable three-fold increase in the minimum inhibitory concentration (MIC) for *S. aureus* and *S. pneumoniae*. Subsequent subculturing of the strains with elevated MICs in antibiotic-free media for 7 days did not result in a reduction of their MIC values for MU1140. Collectively, our findings illustrate the therapeutic potential of MU1140 for management of Gram-positive infections.

Keywords

Lantibiotic; MU1140; Lanthionine; Pharmacodynamics; Antibiotic resistance; MRSA; VRE

1. Introduction

A novel class of antibiotics that has long attracted much attention is the antimicrobial peptides (AMPs). AMPs have emerged as potential therapeutic agents for the treatment of various types of bacterial infections owing to their ability to kill Gram-positive and Gram-negative pathogenic microorganisms and fungi as well as to activate components of the host innate immune system [1–4]. Some of these peptides were also shown to inhibit the replication of enveloped viruses [5]. All AMPs discovered so far share certain similar structural characteristics required for their bioactivity, including an overall positive charge inferred by the presence of multiple arginine and lysine amino acid residues as well as ca. 50% of the peptide's overall primary structure composed of hydrophobic residues [3]. It is thought that these amphiphilic structural features promote binding to and intercalation into bacterial membranes, which then allows the peptide to carry out its antibacterial activity [6].

*Corresponding author. Tel.: +1 386 418 4018 ext. 224; fax: +1 386 418 1660. E-mail address: jhillman@oragenics.com (J.D. Hillman).

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A promising class of AMPs is the lantibiotics. Lantibiotics (lanthionine-containing antibiotics) are peptides with antimicrobial properties that are secreted by certain Gram-positive bacteria [7]. Although to date lantibiotics have not been utilised as pharmaceutical agents, several have been used in commercial applications. Nisin, for example, is a lantibiotic produced by the bacterium *Lactococcus lactis* that has been used extensively as a food preservative since the 1920s.

Lantibiotics are ribosomally synthesised and then undergo extensive post-translational modification. They are characterised by unusual amino acids such as lanthionine (Ala-S-Ala), methylanthionine (Abu-S-Ala), dihydroalanine and dihydrobuterine. MU1140 (mutacin 1140; Fig. 1) is a 22-amino acid lantibiotic produced by *Streptococcus mutans* [8]. It has been extensively characterised with regard to its physical and chemical properties [8,9] and its role in promoting colonisation of the oral cavity by the producer strain. Its unique mechanism of action involves inhibition of peptidoglycan synthesis by binding to and abducting lipid II from its site of action at points of peptidoglycan synthesis [10].

The aim of this study was to evaluate the potential efficacy of MU1140 against a broad spectrum of Gram-positive organisms as well as to assess their potential resistance development to MU1140. Measurement of the minimum inhibitory concentration (MIC) was used to assess the susceptibility of the organisms to MU1140, and time-kill studies provided a dynamic picture of MU1140 antimicrobial action.

2. Materials and methods

2.1. Bacteria and media

Bacterial strains (Tables 1–3) used in the spectrum of activity studies were clinical isolates as well as American Type Culture Collection (ATCC) strains. Kill curves were performed using a multidrug-resistant (MDR) strain of *Staphylococcus aureus* (ONI33) and a MDR strain of *Enterococcus faecalis* (ONI47), both obtained as fresh clinical isolates from Shands Hospital (Gainesville, FL). These studies were also performed using a strain of *Streptococcus pneumoniae* (ATCC 49619). *Staphylococcus aureus* ONI33 and *S. pneumoniae* ATCC 49619 were also used in the development of resistance study. *Staphylococcus aureus* strain ONI33 was shown to be resistant to amoxicillin, ampicillin, cefazolin, cefepime, cefotaxime, ceftriaxone, cefuroxime, cefalothin, ciprofloxacin, clindamycin, erythromycin, imipenem, levofloxacin, meropenem, oxacillin, penicillin, sparfloxacin, ticarcillin, azithromycin, amikacin and chloramphenicol. *Enterococcus faecalis* strain ONI47 was shown to be resistant to ampicillin, ciprofloxacin, erythromycin, levofloxacin, penicillin and vancomycin.

Bacterial strains were stored as 50% glycerol stabs at -80°C . Starter plates of bacterial strains were prepared by inoculation of samples from glycerol stabs onto blood agar plates (BAPs) consisting of 1.5% casein peptone (Remel, Lenexa, KS), 0.5% soy peptone (Remel), 0.5% sodium chloride (Remel), 5% sheep's blood (Lampire, Everett, PA) and 1.5% agar (Fisher, Fairlawn, NJ). *Staphylococcus aureus* strain ONI33 and *E. faecalis* strain ONI47 were grown in cation-adjusted Muller–Hinton broth (Becton Dickinson Biosciences, Franklin Lakes, NJ) at 37°C in a 5% CO_2 incubator. *Streptococcus pneumoniae* strain ATCC 49619 was grown in Todd–Hewitt broth (Becton Dickinson Biosciences) under the same conditions.

2.2. Antimicrobial agents

MU1140 was manufactured by Oragenics Inc. (Alachua, FL). Purity was estimated to be $>90\%$ as determined by analytical reverse-phase high-performance liquid chromatography.

2.3. Susceptibility studies

The MICs of MU1140 against target microorganisms were determined by Focus Bio-Inova (Herndon, VA). MU1140 MIC values for aerobes were determined by the broth microdilution method according to Clinical and Laboratory Standard Institute (CLSI) methodology [11], whilst MU1140 MIC values for anaerobes were determined by the agar dilution according to CLSI methodology [12].

2.4. Time–kill studies

MICs for *S. aureus* strain ONI33, *E. faecalis* strain ONI47 and *S. pneumoniae* strain ATCC 49619 used in the time–kill and development of resistance studies were determined using the broth microdilution method. Inocula were prepared from test organisms grown for 4–6 h in the appropriate broth media and diluted in saline to 0.5 McFarland standard to obtain 100 mL of a starting culture containing 10^6 colony-forming units (CFU)/mL, which was verified by colony counts of replicate samples. Aliquots (10 mL) of the culture were transferred to sterile plastic 25 cm² culture flasks (Corning Inc., Corning, NY) and MU1140 was added from a sterile stock solution to give final concentrations equal to 0.5, 1, 2, 4, 8 and 16 times the MIC for *S. pneumoniae* strain ATCC 49619 and *S. aureus* strain ONI33, and 0.25, 0.5, 1, 2, 4, 8 and 16 times the MIC for *E. faecalis* strain ONI47. Each assay included a growth control tube with no antibiotic.

The cultures were incubated at 37°C and samples were obtained at 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h following addition of MU1140. The samples were washed with phosphate-buffered saline and serially diluted 10-fold in ice-cold normal saline and then 10 µL samples were spotted onto duplicate BAPs. Following incubation at 37°C for 24 h, colonies that arose on plates with 30–300 colonies were counted.

2.5. Development of resistance

Staphylococcus aureus strain ONI33 and *S. pneumoniae* strain ATCC 49619 were grown overnight on BAPs. Cells were scraped from the surface and diluted with saline to 0.5 McFarland standard. Cells were then diluted 1:100 in appropriate broth media to give ca. 10^6 CFU/mL and then 100 µL samples were added to microtitre wells (Corning Inc.) containing 100 µL of doubling concentrations of MU1140 in broth to achieve a final bacterial concentration of 5×10^5 CFU/mL. The microtitre plates were incubated overnight at 37°C in an atmosphere of 5% CO₂. Wells containing the highest concentration of MU1140 that showed turbidity (equivalent to 0.5× MIC) were diluted to 0.5 McFarland standard and used as the inocula to repeat the above process. This process was repeated daily 21 times and the MIC after each subculture was recorded. After the 7th, 14th and 21st repetition, a sample of cells from the 0.5× MIC well was used to inoculate 1 mL of MU1140-free broth, which was grown overnight to saturation. These cells were subcultured in the absence of MU1140 an additional six times, after which MICs for MU1140 were determined using the broth microdilution method.

3. Results

3.1. Susceptibility studies

The results of the Tiers 1 and 2 susceptibility studies are summarised in Tables 1 and 2. The Tier 1 study demonstrated that MU1140 was biologically active against all Gram-positive bacterial strains tested, with MICs ranging from 0.5 µg/mL to 32 µg/mL (Table 1). It was most potent against *Streptococcus pyogenes* (MIC = 0.5 µg/mL) and least potent against *E. faecalis* and *Enterococcus faecium* (MIC range 8–32 µg/mL). MU1140 was not active against Gram-negative bacteria or yeast.

In the Tier 2 studies (Table 2), MU1140 showed greater activity ($\text{MIC} < 8\mu\text{g/mL}$) against *S. pyogenes*, *Listeria monocytogenes* and *Clostridium difficile* than against *S. aureus*, *E. faecalis* and *Bacillus* sp. ($\text{MIC} > 8\mu\text{g/mL}$).

Results of the Tier 3 studies (Table 3) revealed that MU1140 was as active as vancomycin against vancomycin-intermediate *S. aureus*, but was superior to vancomycin against all tested vancomycin-resistant strains of *S. aureus*, *E. faecalis* and *E. faecium*. Vancomycin had lower MIC values compared with MU1140 when tested against all vancomycin-sensitive strains.

3.2. Time–kill studies

One isolate each of *S. pneumoniae* (Fig. 2), MDR *S. aureus* (Fig. 3) and vancomycin-resistant *E. faecalis* (VREF) (Fig. 4) were selected as test organisms for the time–kill analysis. Very similar kill profiles were observed for *S. pneumoniae* and *S. aureus*, characterised by a rapid and significant decline (>3 log drop) in bacterial counts within the first 2 h independent of the antibiotic concentration. Re-growth was observed at lower MU1140 concentrations ($0.5\times$ and $1\times$ MIC for *S. pneumoniae* and $0.5\times$, $1\times$ and $2\times$ MIC for *S. aureus*) but not at concentrations $>8\times$ MIC for all strains. For *S. pneumoniae*, time to 99.9% killing after exposure to MU1140 at $1\times$ MIC and $2\times$ MIC was 5 h, whilst at $4\times$ MIC and $8\times$ MIC it was 2.5 h. For MDR *S. aureus*, time to 99.9% killing after exposure to MU1140 at $0.5\times$ MIC was 1.5 h, and at $4\times$, $8\times$ and $16\times$ MIC it was 0.5 h. The CLSI defines a bactericidal agent as one for which a given concentration reduces the original inoculum by 99.9% ($>3 \log_{10}$ CFU/mL) for each time period, and bacteriostatic if the inoculum was reduced by $0\text{--}3 \log_{10}$ CFU/mL. According to that definition, time–kill studies reveal that MU1140 is bactericidal against *S. pneumoniae* at concentrations $\geq 1\times$ MIC and bactericidal against *S. aureus* at concentrations $\geq 0.5\times$ MIC. The time–kill studies also reveal that MU1140 is bacteriostatic against VREF (Fig. 4) at all concentrations tested and maintained bacterial counts at approximately the initial inoculum size.

3.3. Resistance development study

MIC values resulting from daily subculturing of *S. aureus* strain ONI33 and *S. pneumoniae* strain ATCC 49619 are summarised in Fig. 5. Sequential subculturing of the strains resulted in emergence of variant stains with elevated MU1140 MIC values. The MICs for the parent *S. aureus* was $3.2\mu\text{g/mL}$, which doubled after the second and the twelfth subculturing to stabilise at $12.8\mu\text{g/mL}$. The MIC of MU1140 against the *S. pneumoniae* parent started at $0.4\mu\text{g/mL}$, which doubled with the second, third and fourth subculturing event, stabilising at $3.2\mu\text{g/mL}$. Subculture of the resistant variants in the absence of MU1140 did not affect their respective MICs, indicating that the observed resistance was genetically stable and not an adaptive response.

4. Discussion

The class of antibiotics known as the lantibiotics has been known for decades and throughout this period many investigators (reviewed by Cotter et al. [13]) have predicted their potential for use as therapeutic agents. The goal of this study was to evaluate the lantibiotic MU1140 as a potential antimicrobial agent. Determination of the MICs of MU1140 for select microorganisms was used as a measure of their susceptibility to MU1140. The testing was performed in three stages, in which Tier 1 results indicated that all 30 Gram-positive species tested were sensitive according to CLSI susceptibility breakpoints [14], whilst none of the 28 Gram-negative species or the yeasts tested showed sensitivity. These findings are in accordance with previous studies [8,15]. The lack of sensitivity of Gram-negative bacteria and yeast to MU1140 is likely to be a function of its mode of action. MU1140 exerts its antimicrobial effect by a novel mechanism [16], which involves abduction of lipid II from the plasma

membrane near areas of active peptidoglycan synthesis. The presence of an MU1140-absorbing outer membrane in Gram-negative bacteria and the absence of lipid II in yeast provide explanations for the observed spectrum of activity of MU1140. The results of the Tier 2 study confirmed the effectiveness of MU1140 against multiple strains of selected pathogenic Gram-positive species, including strains resistant to various currently used antibiotics. The results of the Tier 3 study added further evidence for the effectiveness of MU1140 against drug-resistant Gram-positive pathogens. In particular, this study demonstrated the susceptibility of vancomycin- and oxacillin-resistant *S. aureus*, *E. faecalis* and *E. faecium* strains to MU1140. The MICs of susceptible organisms showed a wide range of interspecies variability, with *S. pyogenes* and *C. difficile* being highly susceptible to MU1140 and *E. faecalis* and *E. faecium* being less susceptible. At present there is no definitive explanation for these observed differences.

Although MIC determination is still the gold standard for characterising the potency of an antimicrobial agent, it does not provide information about the time course of the antibiotic's action. This limitation is overcome by the use of time–kill studies [17], which were performed using strains of medically important Gram-positive species, namely *S. aureus*, *S. pneumoniae* and *E. faecalis*. The results of time–kill investigations showed that MU1140 exhibit rapid initial killing against MDR *S. aureus* and *S. pneumoniae*, whereas a bacteriostatic activity was observed against VREF. Vancomycin also exhibits this species-dependent difference in activity [18,19]. MU1140 and vancomycin both target lipid II, but at different moieties on this complex molecule. Thus, it is likely that the involvement of lipid II is important in the observed species-specific differences of MU1140 activity, although the actual basis for this phenomenon remains unknown.

The ability of susceptible microorganisms to develop resistance to MU1140 was tested using an in vitro model. After 21 daily sequential passages in subinhibitory concentrations of MU1140, it was possible to select MDR *S. aureus* and *S. pneumoniae* mutants with modest three-fold elevated MU1140 MICs. This phenotype was stable, indicating the selection of genetic variants. Resistance development to lantibiotics has been extensively studied using nisin (reviewed by Chatterjee et al. [7]) and involved such diverse mechanisms as decreased nisin binding due to changes in the net negative charge of the cell envelope, increased cell wall thickness that altered cell surface hydrophobicity, and the possible existence of inactivating enzymes. In the present study, we have no evidence to support any of these or other mechanisms for the observed modest increase in MICs. This will be the subject of future investigations.

The present studies indicate that MU1140 has a spectrum of activity that includes a number of medically important bacteria. The observed time–kill profiles for certain of these species is consistent with vancomycin, one of the current drugs of last resort, which is currently losing its effectiveness owing to the rise of drug-resistant pathogens. The low-level increase in the MICs of select pathogenic species during repeated cultivation in the presence of sublethal concentrations of MU1140 indicates that development of significant resistance to this molecule will not be easily accomplished. In support of this last contention is the observation that the producer strain *S. mutans* JH1140 has a MIC comparable with other streptococci, indicating that it has not been able to develop effective immunity against its own bacteriocin. JH1140 has been shown to regulate expression of the structural gene for the MU1140 prepropeptide very tightly (Hillman, unpublished data). It is presumed that this regulation allows JH1140 to produce sufficient MU1140 to protect its habitat in dental plaque without affecting its own viability. Additional work is in progress to determine the usefulness of MU1140 as a clinically useful therapeutic agent for the treatment of infectious diseases.

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Fig. 1.
Structure of MU1140.

Fig. 2. Bactericidal activity of MU1140 against *Streptococcus pneumoniae* strain ATCC 49619. ◆, control; ■, 0.5× MIC; ▲, 1× MIC; |, 2× MIC; ▽, 4× MIC; —, 8× MIC. MIC, minimum inhibitory concentration; CFU, colony-forming units.

Fig. 3. Bactericidal activity of MU1140 against multidrug-resistant *Staphylococcus aureus*. ◆, control; ■, 0.5× MIC; ▲, 1× MIC; □, 2× MIC; ▽, 4× MIC; —, 8× MIC; ◇, 16× MIC. MIC, minimum inhibitory concentration; CFU, colony-forming units.

Fig. 4. Bacteriostatic activity of MU1140 against vancomycin-resistant *Enterococcus faecalis*. ◆, control; x, 0.25× MIC; ■, 0.5× MIC; ▲, 1× MIC; □, 2× MIC; ▽, 4× MIC; —, 8× MIC; ◇, 16× MIC. MIC, minimum inhibitory concentration; CFU, colony-forming units.

Fig. 5. MU1140 minimum inhibitory concentrations (MICs) after 21 subculturing events for multidrug-resistant *Staphylococcus aureus* (◆) and *Streptococcus pneumoniae* (▽). Decrease in susceptibility after repeated subculturing in subinhibitory MU1140 concentrations is evident by the increase in the MIC values for MU1140.

Table 1

Tier 1 susceptibility study: MU1140 minimum inhibitory concentrations (MICs) for various Gram-positive microorganisms

Microorganism (no. of isolates)	MIC (µg/mL)
<i>Enterococcus faecalis</i> (3)	16–32
<i>E. faecalis</i> ATCC 29212 (1)	32
<i>Enterococcus faecium</i> (4)	8–32
<i>Staphylococcus aureus</i> (4)	16
<i>S. aureus</i> ATCC 29213 (1)	16
<i>Staphylococcus epidermidis</i> (4)	16
<i>Staphylococcus saprophyticus</i> (2)	4–16
<i>Streptococcus agalactiae</i> (2)	4
<i>Streptococcus intermedius</i> (1)	2
<i>Streptococcus mitis</i> (1)	4
<i>Streptococcus pneumoniae</i> (3)	1
<i>S. pneumoniae</i> ATCC 49619 (1)	4
<i>Streptococcus pyogenes</i> (2)	0.5
<i>Clostridium difficile</i> (2)	1

Table 2
Tier 2 susceptibility study: minimum inhibitory concentrations (MICs) of MU1140 compared with vancomycin for various Gram-positive and anaerobic microorganisms

Microorganism (no. of isolates)	MIC (µg/mL)	
	MU1140	Vancomycin
<i>Enterococcus faecalis</i>		
VAN ^S (9)	16–32	1–2
VAN ^R (9)	16	>64
<i>Streptococcus pyogenes</i>		
ERY ^S	0.5–2	0.5
ERY ^R	0.5–1	0.5
<i>Staphylococcus aureus</i>		
VAN ^S MRSA (9)	8–32	1
Inpatient VAN ^S MRSA (10)	16–32	1
Community-acquired VAN ^S MRSA (4)	16	1
<i>Streptococcus pneumoniae</i>		
PEN ^S (9)	0.5–8	0.25–0.5
PEN ^R (9)	0.25–8	0.25–0.5
<i>Listeria monocytogenes</i> (9)	4	1
<i>Clostridium difficile</i> (9)	0.5–2	NT
<i>Bacillus</i> sp. (9)	16–32	0.5–2

VAN^S, vancomycin-susceptible; VAN^R, vancomycin-resistant; ERY^S, erythromycin-susceptible; ERY^R, erythromycin-resistant; MRSA, methicillin-resistant *S. aureus*; PEN^S, penicillin-susceptible; PEN^R, penicillin-resistant; NT, not tested.

Table 3
Tier 3 susceptibility study: minimum inhibitory concentrations (MICs) of MU1140
in comparison with vancomycin against selected clinical isolates

Microorganism (no. of isolates)	MIC (µg/mL)	
	MU1140	Vancomycin
<i>Staphylococcus aureus</i>		
OXA ^S (22)	2–8	0.5–1
OXA ^R (33)	2–8	0.5 to >128
VAN ^S (51)	2–8	0.5–2
VAN ^I (1)	4	4
VAN ^R (3)	4–8	>128
<i>Enterococcus faecalis</i>		
VAN ^S (17)	4–8	0.5–4
VAN ^R (14)	4–8	32 to >128
<i>Enterococcus faecium</i>		
VAN ^S (12)	2–8	0.5–1
VAN ^R (13)	1–8	64 to >128

OXA^S, oxacillin-susceptible; OXA^R, oxacillin-resistant; VAN^S, vancomycin-susceptible; VAN^I, vancomycin-intermediate; VAN^R, vancomycin-resistant.