PHARMACOKINETICS AND PHARMACODYNAMICS OF THE LANTIBIOTIC MU1140

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To God, my wife, and our family

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Many thanks are due to my advisors, Dr. Derendorf for his insight, advice, and encouragement and to Dr. Hillman for his generosity, support, sense of humor, and the time he took to teach me to write in “English”, I could not have finished this work without you both on my side. Sincere thanks also go to the members of my committee, Dr. Güenther Hochhaus, Dr. Veronica Butterweck, and Dr. Kenneth Rand, as well as Dr. Jeffery Hughes and Dr. Anthony Palmieri.

I also would like to extend special thanks to the post-doc fellows in Dr. Derendorf lab, Sabarinath S and Vipul Kumar for the time they took to answer any of the many little questions that pop along the way. Special thanks are also due to my friends in the department of pharmaceutics, Immo Zdrojewski, Stephan Schmidt, Oliver Grundman, and Matt, and for my friends at OniBiopharma, Inc., Emily McDonnell and Terri Cram for all their help throughout the years. Without your help and support, this would have been much more painful.

Last, but not least, I would like to extend sincere gratitude to my pearl Godien and my son Avanobe, for their encouragement, support, and sacrifices to get me to this point.

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Chair: Hartmut Derendorf
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One of the fastest growing medical concerns is the issue of antimicrobial resistance. Two possible approaches to reduce the emergence of resistance and control bacterial infections are the continuous production of novel antibiotics and selection of appropriate doses and dosing regimens that ensure maintenance of antimicrobial levels at inhibitory concentrations. MU1140 is an antibiotic peptide with a novel mechanism of action that is produced by the bacterium *Streptococcus mutans* JH1140.

In this thesis, an initial evaluation of MU1140 is presented that includes improved production, analysis, and pharmacokinetic and pharmacodynamic assessment. The production of MU1140 has been improved by optimizing MU1140 fermentation conditions and its purification steps. At this point, the optimized yield is 1mg/Liter of fermentation broth. A bioanalytical method for the quantification of MU1140 in rat plasma was developed and validated, with a lower limit of quantitation of 0.39µg/ml. The pharmacokinetic behavior of MU1140 was investigated in Sprague Dawley rats following intravenous administration. The plasma concentration-time profile of MU1140 declined biexponentially with a mean elimination half-life of 1.7 ± 0.1 hours.

In vitro susceptibility to MU1140, determined by MIC screening, showed activity against Gram positive organisms, even in case of drug resistant microorganisms. The *in vitro* pharmacodynamics of MU1140 were further investigated using time-kill studies in a constant concentration *in vitro* model using *Enterococcus faecalis, Staphylococcus aureus,* and *Streptococcus pneumoniae*. MU1140 was shown to act in a bacteriostatic fashion against *E. faecalis* and bactericidal fashion against *S. aureus,* and *S. pneumoniae.* A linked PK/PD model was developed to predict the *in vivo* counts of viable bacterial cells when MU1140 is administered in different dosing regimens.

Collectively these findings illustrate the potential of MU1140 to serve as a therapeutic agent for the management of otherwise difficult to treat infections caused by Gram positive bacteria.

CHAPTER 1
INTRODUCTION

Antibiotic Resistance and Lantibiotics

Antibiotics, also known as antimicrobial agents, are used to fight and control infectious diseases. After their initial discovery in the 1st half of the 20th century they transformed medical care and dramatically reduced illness and death from infectious diseases. However, during the second half of the 20th century, bacteria that antibiotics were able to kill and control began to develop resistance to these antibiotics. After almost fifty years of extensive antibiotic use, disease-causing bacteria are developing resistance to many antimicrobial drugs such that diseases caused by these bacterial are once again causing serious morbidity and mortality. Now, bacterial antibiotic resistance has been called one of the world's most pressing public health problems (1).

Types of Bacterial Resistance

Bacterial resistance to antibiotics can be inherent. For example, *Enterococci* species are inherently resistant to aminoglycosides, *Pseudomonas* species to tetracycline, and Gram negative bacteria are inherently resistant to glycopeptides (50). Inherent resistance could be due to structural characteristics of the cell. A bacterial species could be missing the enzyme targeted by the antibiotic, or have an outer membrane that denies the antibiotic access to its intracellular site of action or on the cell membrane.

On the other hand, previously sensitive bacteria may also acquire resistance to certain antibiotics. The emergence of bacteria that have acquired resistance to the antibiotics they are exposed to is a natural, evolutionary phenomenon resulting from a selective pressure exerted by the antibiotic (50). Acquired resistance could develop by alterations of the microorganism's genetic makeup and acquisition of genes that code for

proteins that confer resistance to the antibiotic (e.g., acquisition of plasmids coding for β-lactamases which degrade β-lactam antibiotics). Transfer of DNA can occur by horizontal gene transfer (HGT). In HGT, genetic material is transferred from one bacterium to another and can occur by one of three mechanisms, transduction, transformation or conjugation (Figure 1-1).

Transduction is the process during which DNA sequences are transferred from one bacterium to another via infections by bacteriophage. During bacteriophage DNA packaging, small pieces of bacterial DNA are incorporated into the viral capsid which is injected into the next susceptible bacterium. The DNA conferring resistance can either by incorporated into the bacterium genome by recombination or into a plasmid. Phenotypically, the recipient bacterium is now resistant to the antibiotic.

In transformation, free DNA is up-taken by the bacteria from the environment. This free DNA is released into the external environment after the death of its carrier and that DNA is "collected" by other bacteria, which integrates it into their genome by recombination, or it might exist as plasmid (20).

The last mechanism is conjugation, where direct cell-cell contact between two bacteria lead to the transfer of plasmid or chromosomal DNA from the donor to the recipient bacterium. This mechanism accounts for most of the resistance emergence among bacterial populations (20).

Due to the relatively low inter-species specificity and ease of these DNA acquisition methods, transfer of genes encoding resistance from one microbe to another or from one species to another caused an explosive emergence and spread of antibiotic resistance bacteria (20). Such acquired resistance may be manifested through one of

many mechanisms (Figure 1-2). Spontaneous mutations in the gene encoding the protein targeted by the antibiotic leading to the alteration of the antibiotic binding site and inability of the antibiotic to interact with its site of target molecule and exert its antimicrobial effect (3). Genes may encode transporter proteins that function to pump the antibiotic out of the cell. The development of alternative metabolic pathways to those inhibited by the drug is also a common physiological mechanism of resistance resulting from alteration in the cell's genome either by mutations or gene transfer by plasmid (50). But, most common are plasmids encoding enzymes that degrade and inactivate the antibiotic molecule once it is inside the bacterial cell (e.g. as mention above, the β-lactamases which degrade all β-lactam antibiotics).

Bacterial Resistance and Future Prospects

Medically, the most worrisome drug resistant bacteria are the multidrug resistant Gram-negative (e.g. *Pseudomonas*, *Enterobacter*, and *Salmonella* species) and Gram positive organisms (*Staphylococcus*, *Enterococcus*, and *Streptococcus* species) (3). Unfortunately antibiotic resistance is an unavoidable consequence to any new antibiotic that bacteria get exposed to. This in turn leads to the rapid rise in bacterial populations that are resistant to developed antibiotics, and the subsequent stress to society in terms of morbidity, mortality, and increased health care expenditures. For all the mentioned reasons, our policies regarding production, handling, and usage to antibiotics has to evolve. Less use of antibiotics, appropriate choice of antibiotic and dosing regimen, and the discovery and production of new antimicrobials are crucial factors to contain the problem in hand (1). Given the decreasing utility of available antimicrobials, attention to development of new antimicrobials is becoming increasingly important (4).

The Potential of Antibacterial Peptides as Therapeutic Agents for Human Use

Antimicrobial peptides have emerged as potential therapeutic agents for the treatment of various types of bacterial infections due 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 (6, 9, 34, 90). Some of these peptides were also shown to inhibit enveloped viruses replication (57). So far, all discovered antimicrobial peptides share unique structural characteristics required for their bioactivity, which include:

1- An overall positive charge produced by the presence of multiple arginine and lysine amino acids,

2- Approximately 50% of the peptide's overall primary structure is composed of hydrophobic residues. This high hydrophobicity forces these peptides to assume an amphiphilic conformation when binding to bacterial membranes, thus allowing intercalation and subsequent perforation or penetration of the cell membrane into the cytoplasm (69).

Antimicrobial Peptides Modes of Action

Antimicrobial peptides can exert their effect either by interaction and perforation of the plasma membrane or by penetration through the membrane and binding to intracellular targets which can result in inhibition of protein or DNA synthesis, cell division, or induction of autolysis. The cationic nature attracts the antimicrobial peptides to negatively charged bacterial membranes rich in anionic phospholipids such as phosphatidylserine, anionic teichoic acids of Gram positive bacteria, and negatively charged lipopolysaccharides (LPS) of Gram negative bacteria. Upon interaction with the bacterial membranes, these cationic peptides displace divalent cations from the membrane leading to membrane destabilization, which enables the peptide to translocate through the membrane, a mechanism known as self-promoted uptake (69). Other

antibiotic peptides are known to function as pore formers (13) which perforate the bacterial plasma membrane, or inhibitors of bacterial cell wall synthesis via a mechanism known as lipid II abduction (35) where the antibiotic removes lipid II from its site of activity thus disrupting cell wall synthesis.

Attractive Attributes of Antimicrobial Peptides

One of the prominent features of these peptides is that they can kill multidrug resistant bacteria at concentrations comparable to conventional antibiotics, but they kill at a much rapid rate (8). Another attractive attribute is the fact that there is no cross resistance between the currently used antibiotics and these peptides. Hence methicillin-resistant *S. aureus,* multidrug resistant *P. aeruginosa*, as well as other multidrug resistant bacteria are susceptible to these antimicrobial peptides at safe concentrations (91), making these peptides an attractive solution to the current problem of bacterial antibiotic resistance.

The major hurdle preventing large scale exploitation of these peptides for human use is the high cost of manufacturing of antimicrobial peptides and inability to purify these molecules to homogeneity (15).

Lantibiotics

A unique class of antimicrobial peptides that has gained increased attention in the last decade is the lantibiotics. Lantibiotics were discovered the year before Fleming discovered penicillin. In 1928, L. A. Rogers mentioned a potent antimicrobial substance produced by certain lactic acid bacteria, but the nature of the inhibitory compound was not known at the time (71). Lantibiotics are peptides that function as bacteriocins. Lantibiotics are characterized by the presence of thioether bridged amino acids known as lanthionine (Lan) and/or methyllanthionine (MeLan), as well as other modified amino

acids such as didehydroalanine (Dha) and didehydrobutarine (Dhb) (Figure 1-3 A). Lantibiotics are ribosomally synthesized and posttranslationally modified to their biologically active forms which are active mainly against Gram positive bacteria. The word lantibiotic was first coined in 1988 as an abbreviation for lanthionine-containing antibiotic peptide. The defining posttranslational modification of lantibiotics is that they contain the lanthionine (Ala-S-Ala) or ß-methyllanthionine (Abu-S-Ala) (13).

Lantibiotic Classifications

Lantibiotics are classified as type A or B based on their ring structure as well as their biological activity. Type A lantibiotics are cationic peptides of 20-34 amino acids in length and assume an elongated and amphipathic state in physiological solution. Further, within the Type A lantibiotics, those which are posttranslationally modified by the action of a dehydratase enzyme (LanB) and a cyclase enzyme (LanC) are classified as Type AI, while those that are dehydrated and cyclized by a single enzyme (LanM) are classified as type AII. Type B lantibiotics on the other hand are globular and compact in configuration with no net charge or anionic at pH 7 (13).

Lantibiotics Biosynthesis

Lantibiotics' primary structure is encoded in a gene that is part of an operon containing other genes that encode for enzymes required for the posttranslational modification, processing, and translocation of the mature lantibiotic (Figure 1-3 B). The first gene, LanA, encodes the peptide precursor preceded with an N-terminal leader sequence followed by the propeptide from which the mature lantibiotic is produced. Encoded in the same operon are LanB and LanC. LanB codes for a dehydratase which dehydrate serine and threonine amino acids of the propeptide to form didehydroalanine (dha) and didehydrobutarine (dhb) residues respectively. LanC codes for a cyclase

enzyme that catalyze the interaction of the dehydrated residues with sulfhydryl groups of Cysteine residues to form the lanthionine amino acids. The lantibiotic operon also contains a LanT gene which codes for a transporter of the precursor and inactive lantibiotic to the extracellular environment. At his point, the lantibiotic is inactive due to the presence of the N-terminal leader peptide. LanP encodes a protease that is anchored to the extracellular leaflet of the plasma membrane of the producer cell. LanP protease will cleave the leader sequence liberating the mature and fully functional lantibiotic (13).

MU1140

A member of the Type A1 lantibiotics is MU1140 (Figure 1-4). MU1140 is naturally produced by a strain of the common oral bacterium, *Streptococcus mutans*. The prototype of type A lantibiotics, nisin, has been developed as a food preservative which has been given the "generally recognized as safe" status by the FDA.

The primary mode of bactericidal activity of type A lantibiotics is believed to be disruption of the cytoplasmic membrane, causing the efflux of ions and metabolites and desynergization of the target cell (13). However, recent data suggest that this may not be the mode of bactericidal activity of MU1140 (35).

MU1140 Mechanism of Action

MU1140 acts to inhibit cell wall synthesis by a novel mechanism known as "Lipid II Hijacking". Lipid II hijacking (Figure 1-5) involves removal of the bacterial cell wall subunits carrier, lipid II, from its site of action on the bacterial cell membrane at the cell division septa (35) and aggregation to a different nonfunctional site.

Lipid II (Figure 1-5 B) is a key molecule in bacterial peptidoglycan synthesis (Figure 1-5 A). Lipid II functions to transport cell wall subunits from the cytoplasm, their site of biosynthesis, across the bacterial cytoplasmic membrane to the periplasmic

space, where they are assembled into the growing peptidoglycan polymer. Lipid II is synthesized in the cytoplasm and is composed of N-acetyl glucosamine-N-acetyl muramic acid-pentapeptide units linked to an undecaprenyl lipid tail by a pyrophosphate. The assembled Lipid II transposes itself across the membrane where the sugar subunits are transferred to the growing peptidoglycan chains and the isoprenoid carrier is recycled back to the cytoplasm where it picks up more peptidoglycan subunits and so on.

MU1140's Three Dimensional Structure

Elucidation of the native three dimensional structure of a molecule can provide us with an insight into the physiological role the molecule assumes in its habitat. In the case of MU1140, a knowledge of the three dimensional structure could allow for a better understanding of its antimicrobial activity, as well as the knowledge to manipulate and improve the molecule's properties. Like nisin and gallidermin, the thioether ring structures of MU1140 were found to be rigid and well defined, and like nisin the regions not spanned by thioether linkages were quite flexible (83). This motif of two domains fixed by lanthionine rings joined by a flexible "hinge" seems to be a common feature of Type A lantibiotics. The molecule has an overall horseshoe-like shape kinked at the "hinge region" between rings B and C (Figure 1-6). The thioether bridges that form the ring structures of the molecule are exposed to the surface. Thioether bridges are resistant to enzymatic or non-enzymatic oxidation/reduction activities that would otherwise easily open disulfide bonds. The hinge region contains a potentially susceptible arginine at residue 13 that appears to be sterically protected. The hinge region, quite flexible, may be important for bactericidal activity by allowing the lantibiotic to orient properly in the membrane and for membrane insertion properties of the molecule (53, 54).

Pharmacokinetic and other Essential Considerations for Successful Antibiotic Therapy

Antimicrobial therapy is a complex process and its success is dependent on the appropriate selection of the right antibiotic and the right dosing regimen. Many factors are involved in the process of antimicrobial chemotherapy. There are infective agent-associated factors like the bacteria's susceptibility to the available antibiotics as well as the infection site. There is also host-related factors which include the immune status and the patient's overall physiological state which could dramatically affect the antibiotics' ADME (absorption, distribution, metabolism, and excretion) processes (24).

The ADME process, also referred to as the drug's pharmacokinetics or drug's disposition in the body, is generally depicted as what the body does to the drug (21). The most important pharmacokinetic parameters that are used to predict the probability of therapy's success are the peak serum level (C_{max}), the trough (C_{min}), and the area under the serum-concentration time curve (AUC) (47). Serum levels of an antibiotic need to be above a certain level during the dosing interval to eradicate the target microorganism and prevent the emergence of resistant strains (44). Pharmacokinetic (PK) studies will measure the time course of the antibiotic in the body.

Antibiotics Mode of Action

Antibiotics can exert their antimicrobial effect via one or more of multiple mechanisms. Shown in Figure 1-7 examples of certain antibiotics and their mode of action. The aminoglycosides and chloramphenicol bind and inactive bacterial ribosomes thus inhibiting protein synthesis resulting in bacterial cell death. Beta-lactam antibiotics (which include the penicillins, cephalosporins, carbapenems and monobactams) and glycopeptides, include vancomycin, act by inhibition of cell wall synthesis via prevention

of cross-linking of N-acetylglucosamine and N-acetylmuramic acid cell wall subunits, which renders the bacterial cell susceptible to osmotic lysis. The quinolones and rifampin inhibit DNA and RNA synthesis, respectively. Trimethoprim and sulfonamides function by blocking cell metabolism via inhibition of folic acid biosynthesis.

Pharmacodynamics is the study of the relationship between the antibiotic's concentration and its effect. When studying antibiotic pharmacodynamics, two patterns of killing behavior emerge: Concentration-Dependent and Time-Dependent killing (33). More specifically concentration-dependent antibiotics (e.g. aminoglycosides, daptomycin, and the fluoroquinolones) have activity that is proportional to the antibiotic concentration in the medium, and thus the goal of therapy when using a concentration-dependent antibiotic is to achieve a large peak serum concentration (C_{max}) since the greater the concentration the faster and more complete bacterial eradication is achieved. Examples of Time-Dependent Antibiotics are the carbapenems, cephalosporins, and penicillins. For these antibiotics, the extent of killing is dependent on how long they persist in the medium. Thus, the extent and duration of exposure needs to be maximized during therapy to ensure the success of therapy.

Using this classification, the clinician would design a dosing regimen that is optimized according to the type of bacteria and antibiotic pair to be used. Using an unoptimized dosing regimen may lead to failure of antibiotic therapy or, even worse, the emergence of bacterial subpopulations that have developed resistance to the antibiotic in use. Thus, the selection of the appropriate dosing regimen is critical to successful therapy (73). Historically, the process of choosing an antibiotic dosing regimen has been based on trial and error or by following a dosing design of another drug that "worked". This

approach is subject to high chance of failure since it does not take into account differences between drugs and their mechanisms of action, as well as genetic differences between individuals, effect of difference disease states on patient's physiology and drug metabolism capabilities (49). The new and logical approach to dosing of an antibiotic and regimen design is based on pharmacokinetic and pharmacodynamic properties of the drug in a specific host (38). Due to the increase of microorganism resistance to currently used antibiotics, PK/PD modeling for antibiotic dosing regimen design is becoming an increasingly critical aspect of the antibiotics' development process. By incorporating pharmacokinetic and pharmacodynamic data obtained from in vitro as well as animal data into a mathematical PK/PD model , it is suitable to develop a rational recommendations that involve the right drug and the right dosing regimen to ensure clinical success of therapy (21). Several PK/PD modeling approaches have been devised for the optimization of antimicrobial therapy. These approaches are based on either the minimum inhibitory concentration (MIC) of the antibiotic or on the time-kill behavior as measured by time-kill studies.

PK/PD Modeling - MIC

The minimum inhibitory concentration (MIC) is defined as the minimum amount of antibiotic needed to inhibit growth of an initial bacterial inoculum of $1x10^5$colony forming units (cfu)/ml as is measured by lack of visible growth after 18-24 hours. The MIC of an antibiotic is currently the most widely used measure of antimicrobial activity.

There are three MIC-based PK/PD indices (Figure 1-8) commonly used to predict and simulate the activity of an antibiotic. They are (i) the ratio of peak plasma concentration (C_{max}) to MIC (C_{max}/MIC), (ii) the time antibiotic plasma concentration is

above the MIC (T>MIC), and (iii) the ratio of the area under the plasma concentration-time curve to MIC (AUC/MIC).

C_{max}/MIC is the quotient of the maximum attained plasma concentration by the MIC of the antibiotic for that specific bacterial species. To ensure success of therapy and prevent emergence of resistant populations, a C_{max}/MIC ratio of 10 or higher is the target when designing the dosing regimen (63).

T>MIC is the duration when the plasma concentration of the antibiotic is above the MIC of that antibiotic against that specific bacterial species. When designing a dosing regimen, the target is to achieve antibiotic concentration that exceeds the MIC more than 50% of the dosing interval (63).

AUC_{24}/MIC is the quotient of the attained area under the curve (AUC) of the antibiotic plasma concentration over a 24 hour period divided by the MIC. This index provides information on the total exposure of the body to the antibiotic. For Gram negative bacteria, a ratio of >125 is a good indication of efficacy and therapy success, and a ratio of >50 is used for Gram positive bacteria.

PK/PD Modeling - Time Kill Studies

Time-kill studies are frequently conducted by inoculating several vented cap flasks containing the appropriate media with the pathogen at mid-log phase for a final bacterial concentration of 1-10$\times 10^5$ cfu/ml. Different concentrations of the antibiotic are added to the flasks. Samples of the culture are removed at different time points, spotted on suitable agar media plates and incubated over night at optimal conditions of growth. Viable cells/colonies are counted to determine the number of viable cells at different time points. Data is plotted as log cfu/ml vs. time and all antibiotic concentration are plotted on the same chart. Data from the time-kill curves at different concentrations is translated

to a mathematical formula that will predict the viable bacterial cell count when different doses and dosing regimens of the antibiotic are used.

The incorporation of the drug's PK and PD data into a single model allows the use of simulation techniques to predict the effect time profile resulting from a certain dosing regimen (Figure 1-9).

Conclusion

The current antibiotic resistance crisis dictates the need for new antibiotics with novel mechanisms of action. Antimicrobial peptides, particularly lantibiotics, are attractive candidates to satisfy the current unmet medical need due to their broad spectrum of activity, antibacterial action, and the lack of cross-resistance to currently prescribed antibiotics. MU1140, a bacteriocins produced by *Streptococcus mutans*, is a lantibiotic with promising prospects for the control of Gram positive infection. The small size, good stability, low immunogenicity, and broad spectrum of activity of MU1140 make this molecule an extremely attractive candidate for use as a therapeutic agent for human use.

Further studies are required to characterize the PK/PD relationships of MU1140 in animal models. This data is required for MU1140 optimal dosing to ensure success of therapy and reduction of the probability of resistant strains emergence.

Hypothesis and Objectives

The goal of this work was to investigate the applicability of MU1140 as an antimicrobial agent for human use. Based on early discovery data, MU1140 shows activity against Gram positive microorganisms and hence is a promising antibiotic for treatment of a variety of Gram positive infections. In preparation for an IND submission, a reproducible production and purification techniques that yield high quality MU1140

had to be established, investigation of the pharmacokinetics of MU1140 in the rat model, as well as thorough understanding of the pharmacodynamic of this new chemical entity must be achieved.

The specific aims of this work are:

(1) Improvement of Streptococcus mutans strain JH1140 Production of MU1140 by Fermentation and Purification

(2) Development and Validation of an Extraction and LC-MS Quantification Method for the Lantibiotic MU1140 in Rat Plasma

(3) Pharmacokinetic and Pharmacokinetic/Pharmacodynamic Evaluation of the Lantibiotic MU1140 in Sprague Dawley Rats

(4) In Vitro Pharmacodynamic Activity Assessment of the Lantibiotic MU1140

(5) Assess the degree of In Vitro Serum Proteins Binding and its Effect on the Pharmacodynamics of the Peptide Antibiotic MU1140

This dissertation will be presented as a compilation of manuscripts addressing the specific aims stated above.

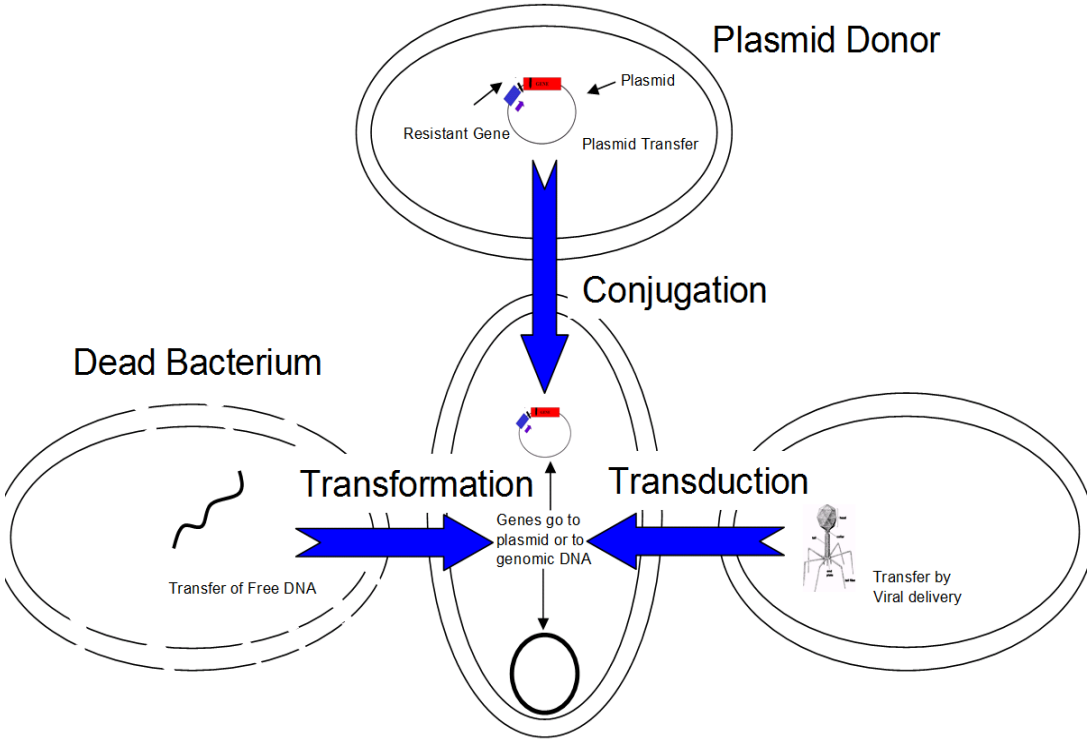


Figure 1-1. Lateral or horizontal gene transfer (HGT) (20).

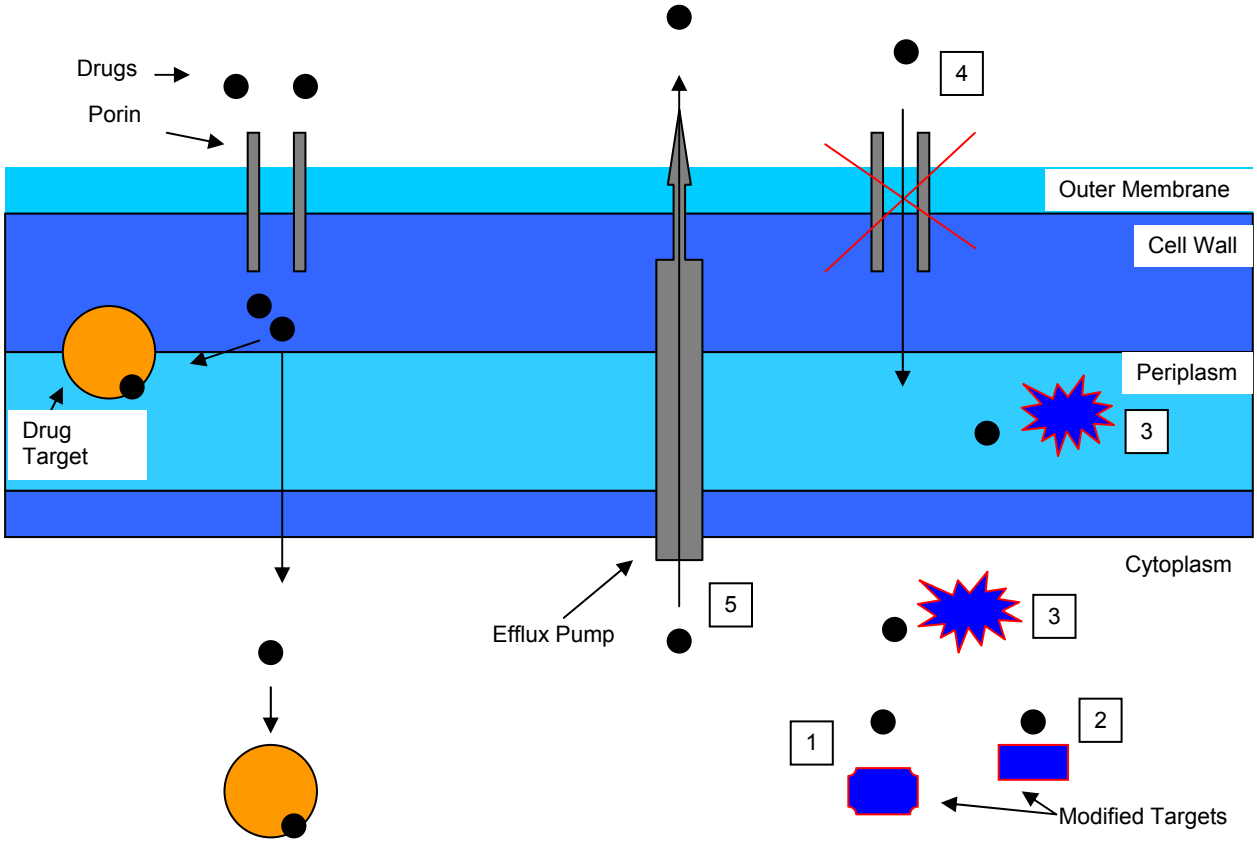


Figure 1-2. Different mechanisms of bacterial antibiotic-resistance. A Sensitive bacteria uptakes the antibiotic. Antibiotic binds to target and exerts it antimicrobial effect. B. Possible resistance mechanism: (1) Alteration of target, thus antibiotic looses effect. (2) Acquisition of a modified target that the antibiotic will not bind to. (3) Enzymatic inactivation of the antibiotic, this is the most common antibiotic resistance mechanism, where an existing enzyme processes the antibiotic and modifies it so that it no longer affects the microorganism. Reduction of the cytoplasmic concentration of the antibiotic, either by (4) mutations of transporters that bring the antibiotic inside the cell, or (5) Actively pumping the antibiotic outside (20).

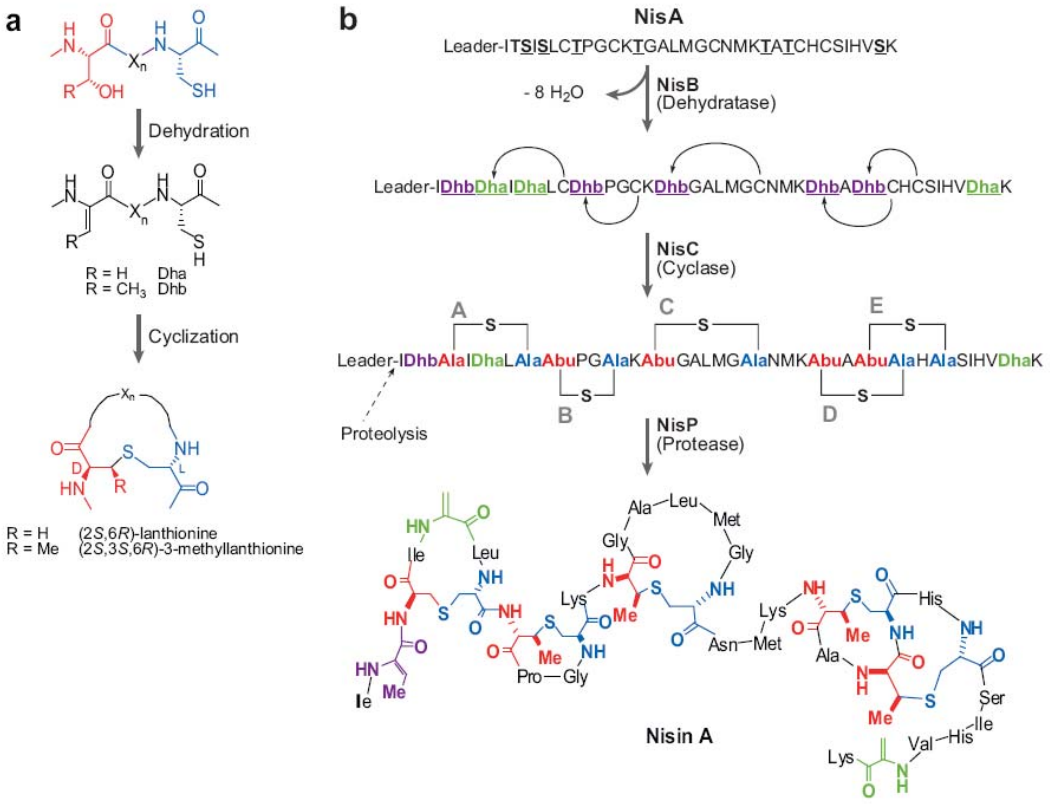


Figure 1-3. Lantibiotic maturation process. A. Dehydration of Ser and Thr residues to produce Didehydroalanine (Dha) and didehydrobutyrine (Dhb) respectively and the formation of the thioether bridge to yield a mature lanthionine. B. The process of Lantibiotic Biosynthesis. Lantibiotic posttranslational maturation process exemplified by the lantibiotic Nisin. The NisA prepeptide is composed of N-terminal leader sequence attached to the active moiety. NisB catalyzes the dehydration of Ser and Thr residues, while NisC catalyzes the cyclization reaction by addition of sulfhydryl groups of Cys residues to Dha and Dhb residues to generate the cyclical thioether bridges. After completion of the dehydration/cyclization reaction the protease NisP proteolytically removes the leader sequence to produce the mature Nisin. Reprinted, with permission, from the Annual Review of Microbiology, Volume 61 (c) 2007 by Annual Reviews www.annualreviews.org (13, 48).

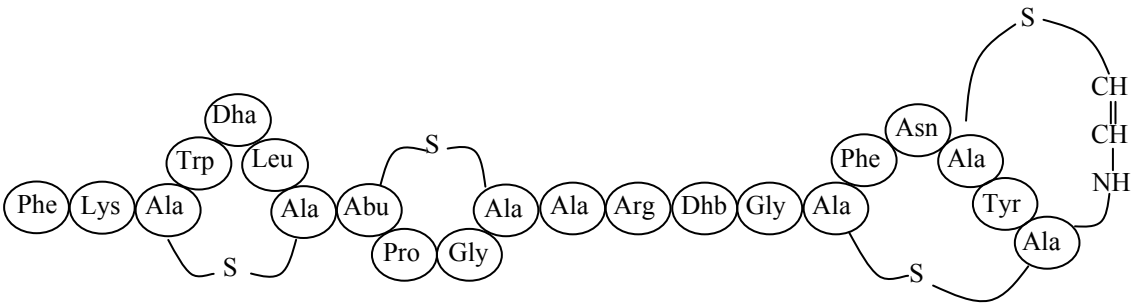


Figure 1-4. The mature MU1140. Abbreviations: dha, 2,3-didehydroalanine; dhb, 2,3-didehydrobutyrine; abu, 2-aminobutyric acid (37).

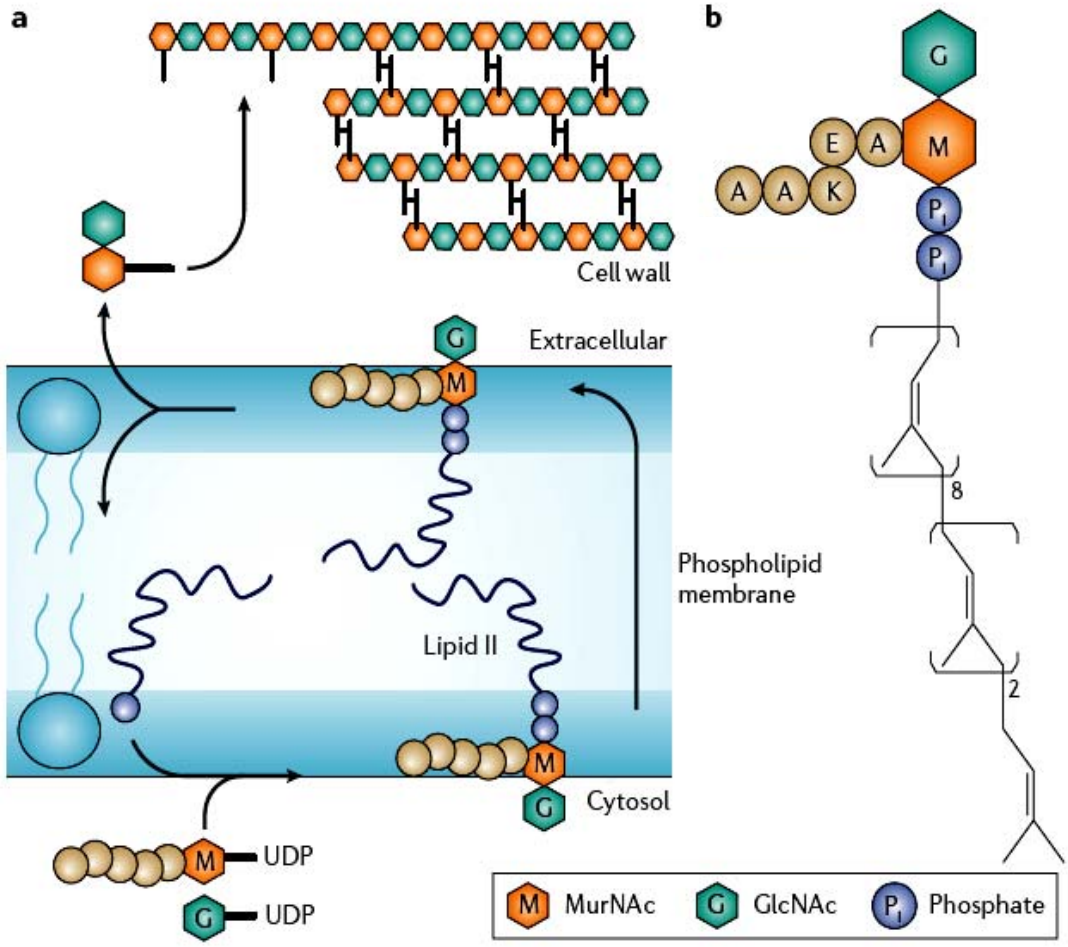


Figure 1-5. Role of lipid II in bacterial cell wall biosynthesis (7). Reprinted with permission from Nature Publishing Group.

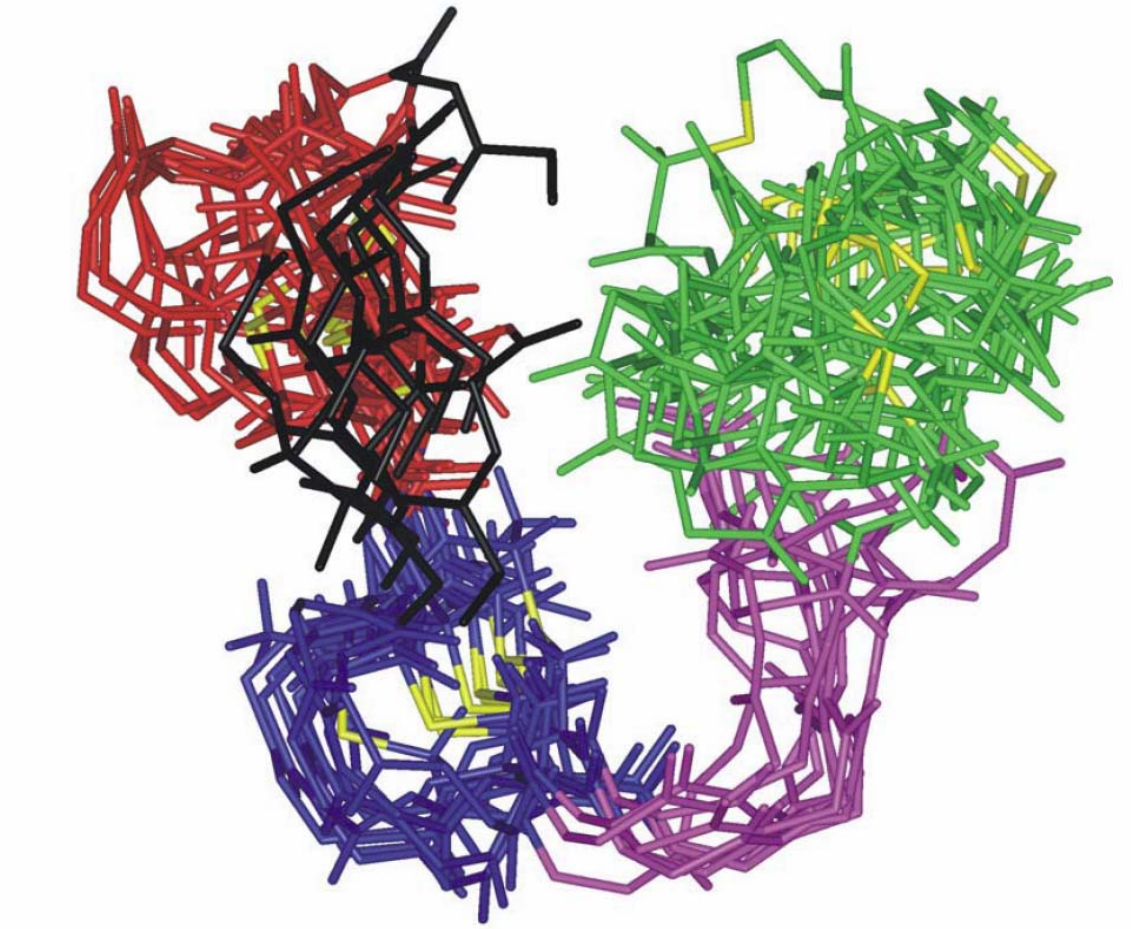


Figure 1-6. MU1140 three dimension structure as determined by NMR. The overall backbone structure of MU1140 assumes a horseshoe shape. Rings A and B are on one side while the intertwined rings C and D are positioned on the other side. Ring A (red), Ring B (blue), Hinge (purple), Ring C and D (green) and thioether linkages (yellow) are visible. The first two N-terminal amino acids, Phe1 and Lys2, are shown in black (77).

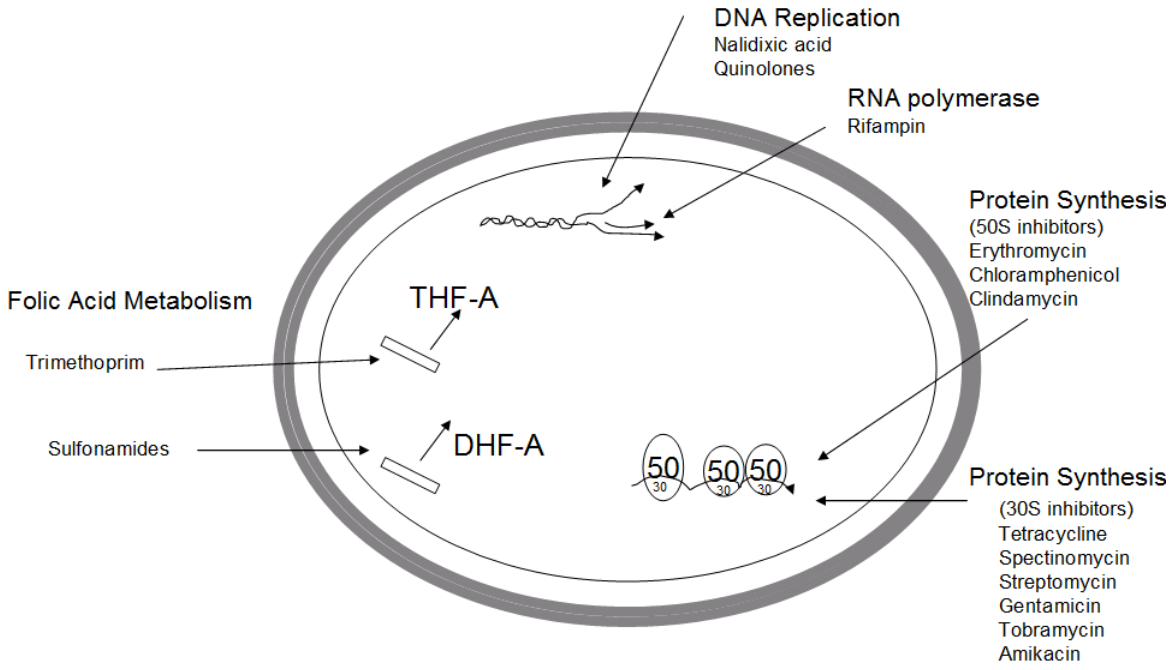


Figure 1-7. Representative antibiotics and their mode of action (59).

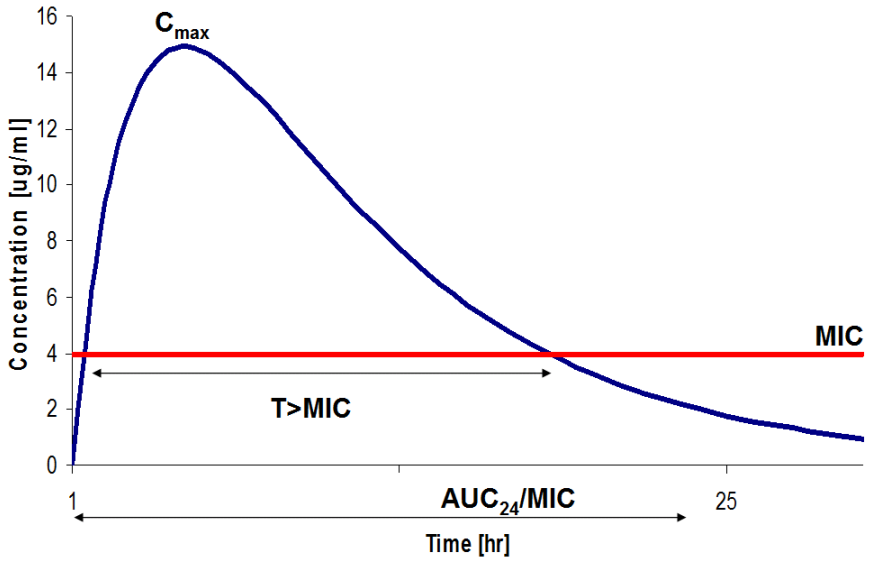


Figure 1-8. MIC-Based Pharmacokinetic and Pharmacodynamic Indices (55)

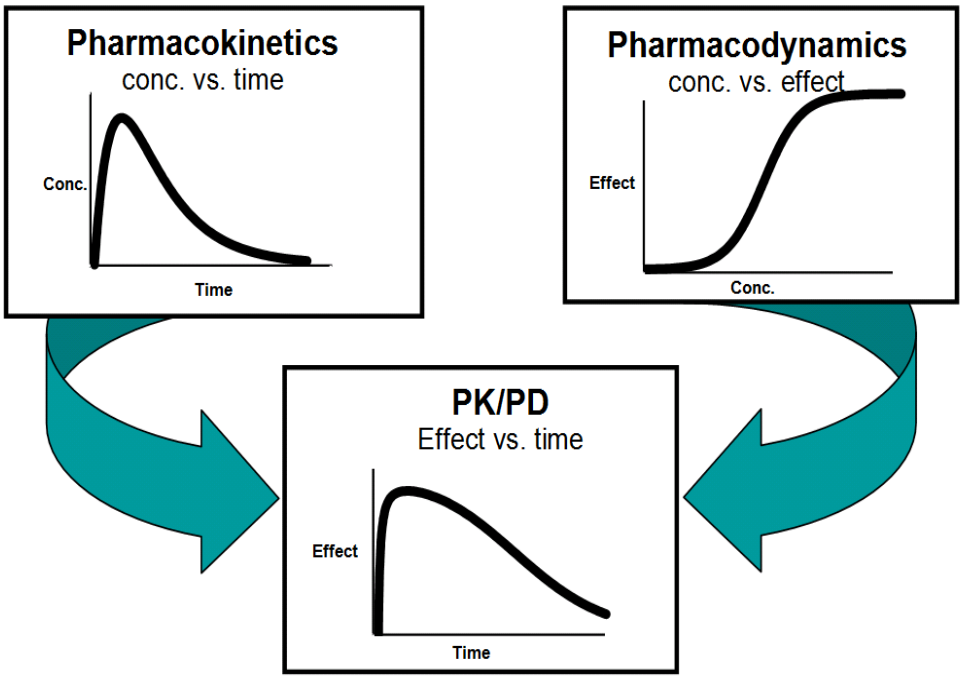


Figure 1-9. PK/PD modeling as a combination of the two classical pharmacology disciplines pharmacokinetics (PK) and pharmacodynamics (PD) (51).

Table 1-1. Current classification of Bacteriocins (13).

class	characteristics	size	subclasses
Class I	posttranslationally modified peptides containing (methyl)lanthionines (lantibiotics)	< 5 kDa	Type A: elongated shape
			Type B: globular shape
Class II	heat-stable peptides of 37–58 amino acids; leader peptide removed during maturation	<10 kDa	Type IIa: N-terminal consensus YGNGVXC, *Listeria*-active, contain 1–2 disulfides
			Type IIb: two-peptide systems
Class III	heat labile, large proteins	>30 kDa	

CHAPTER 2
IMPROVEMENT OF *STREPTOCOCCUS MUTANS* STRAIN JH1140 PRODUCTION OF MU1140 BY FERMENTATION AND PURIFICATION

In this study, the effects of various parameters on the fermentative production of MU1140 by *Streptococcus mutans* strain JH1140 was tested and optimized using the "Sixfors" fermentor. The parameters we evaluated were the fermentation medium composition, oxygen tension, pH, and inoculum size. The highest MU1140 production was achieved using a fermentation medium that consisted of 5% yeast extract, 0.5% $CaCl_2$ and 4% glucose under microaerophilic conditions. pH is to be maintained at a constant 5.1 ± 0.1 by the addition of 5N NaOH. An inoculum size of 10% was used and fermentation lasted 24 hours. The purification method consisted of a precipitation step using ammonium sulfate to precipitate the bioactivity followed by selective uptake in 80% IPA and two separation steps using C18 reverse phase chromatography where the activity is eluted first using an acetonitrile gradient followed by a second run that used a methanol gradient. The activity-containing fraction was lyophilized and > 92% pire MU1140 was recovered as fluffy white strands.

Introduction

Antimicrobial therapy was revolutionized by the introduction of antibiotics in the 1930s and millions of lives have been saved since. Soon after their introduction, some broad spectrum and regularly prescribed antibiotics lost their ability to control certain bacterial infections (10), and currently more 70% of nosocomial infections are caused by drug resistant bacteria (1, 9). Annually, infections caused by *Staphylococcus aureus* alone, which represent 16% of infections nationwide, results in 12,000 inpatient deaths, 2.7 million days in excess length of stay, and 9.5 billion dollars in extra hospital charges (11).

Two factors contribute to the continuous exacerbation of this health crisis. The first is the diminishing antibiotics pipeline (15), especially those with novel mechanisms of action. According to Rice *et al*, since 1998, only ten antibiotics have been approved; out of which only two possess a novel mechanism of action. The other factor is the continuous and rapid emergence and spread of new bacterial strains that are resistant to currently used antibiotics due to unoptimized dosing regimens (14). Thus, there is a need for new antibiotics and appropriate dose design can not be over emphasized.

A class of antibiotics that is lately gaining much attention is the lantibiotics (2). These antibacterial peptides are produced by Gram positive bacteria and released into the environment to destroy organisms that compete with the producer strain for its ecological niche. Lantibiotics are ribosomally synthesized and posttranlationally modified to incorporate unusual amino acids such as lanthionine (Lan, ala-S-ala), methyllanthionine (MeLan, abu-S-ala), didehydroalanine (Dha) and didehydrobuterine (Dhb). MU1140 is a 22 amino acid lantibiotic that is produced by *Streptococcus mutans* strain JH1140 (7) and its novel mechanism of action, dubbed "Lipid II Hijacking", involves disruption of cell wall synthesis (6, 16). MU1140 is active against Gram positive bacteria including species of medical importance such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE) (4). Unfortunately, for the last 50 years lantibiotics have been utilized to a limited extent as food preservatives (3), to treat peptic ulcers caused by *Clostridium difficile* and *Helicobacter pylori* (5), and to treat acne caused by *Propionibacterium acnes* and bovine mastitis cause by *S. aureus* (12). The major obstacle to the clinical development of lantibiotics is their very low production titer and/or inability to be purified to homogeneity (17). In this study,

MU1140 production and purification procedures developed at Oragenics, Inc. are described.

Material and Methods

Media and Reagents

Yeast extract (YEX), Todd Hewitt Broth (THB), Tryptone Soy Broth (TSB), Luria-Bertani (LB) broth, Brain Heart Infusion (BHI) Broth, Tryptic Soy (TS) Broth, were purchased from Difco Laboratories (Detroit, MI, USA). Acetonitrile, isopropyl alcohol, triflouroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO).

Bacteria and Starter Culture

Stock cultures of *S. mutans* strain JH1140 and Micrococcus luteus (ATCC 272) were kept at -80^{o}C in 30% glycerol TSYEX (3% tryptic soy broth and 0.3% yeast extract). Six hours prior to the fermentation reaction, an inoculum was grown at 37 °C in TSYEX. The bacteria are grown for 5 hrs to an OD of 1.1 read at 600nm. This starter culture was grown in 2 liter Erlenmeyer flasks containing 500 ml of media at 37^{o}C with shaking at 200 rpm.

Study Design

The "Sixfors" fermentor (Infors, Bottmingen, Switzerland; figure 2-1) is composed of six vessels that can be controlled independently. Different cultivation parameters, namely, fermentation medium, carbon and nitrogen source, salt content, oxygen tension, pH, and inoculum size, were varied and the subsequent effect on MU1140 titer was quantified using a delayed antagonism bioassay using *M. luteus* (ATCC 272) as the susceptible strain. This process was repeated until conditions resulting in the optimal MU1140 concentration were identified. During the optimization process, biomass formation was determined by centrifugation of culture and determining the weight of the

cell mass. For determination of MU1140 titer, the supernatant of the culture broth was analyzed by bioassay.

Medium Composition

Different commercially available media were used in pilot fermentation processes to identify a medium with MU1140 inducing capability. Once identified, the components of the medium were varied to determine the optimal mixture for MU1140 production.

Oxygen Tension

The production of MU1140 as a function of dissolved oxygen in the culture medium was optimized by varying the culture stirring speed and sparging with nitrogen in the fermentation vessel. Oxygen tension was monitored using an O_2 probe and the amount of dissolved oxygen was varied between 0.1% to 100% saturation throughout the fermentation period. Samples of the culture liquor were sampled for their MU1140 titer and biomass content.

pH and Inoculum Size

Fermentation medium pH was controlled by the automatic addition of 2M NaOH and was varied between pH 4 - 7.5. The MU1140 titer was measured. We also investigated the effect of inoculum size on the final MU1140 titer. Inoculum size was varied between 0 - 100% of the total fermentation volume.

Delayed Antagonism Assay

Stock cultures of *M. luteus* were maintained at -80^{o}C in 30% glycerol TSYEX. The assay involved growing a culture of *M. luteus* to optical density (600 nm) of 0.2 in TSYEX. The culture was diluted 1:5 with molten top agar (3% tryptic soy broth with 0.75% agar) maintained at 45°C, and spread over the surface of a Trypticase Soy agar plate. Samples containing MU1140 activity to be tested were serially diluted in 50%

acetonitrile and 5µl samples were spotted and allowed to air dry. After incubation overnight, the inverse of the highest dilution producing a visible zone of clearing and was the MU1140 titer.

HPLC Column, Purification Conditions

The MU1140 purification scheme used a number of steps. The culture was centrifuged for 15 minutes at 8000 ×g at room temperature to remove the *S. mutans* cells. Antimicrobial activity was precipitated from the culture supernatant by slow addition of ammonium sulfate to a final concentration of 30% (w/v), followed by overnight incubation at 4^{o}C. The precipitated material was recovered by centrifugation (8,000xg for 20 minutes at 4^{o}C). MU1140 was extracted from the recovered precipitate using 3 treatment of 80% isopropyl alcohol for 24 hours each. The pH adjusted to 3.0 with 12N HCl, and the sample was stored at 4^{o}C until used. The isopropanol was removed and two volumes of 40% acetonitrile were added. The material was passed through a 0.22 µ filter prior to reverse phase separation and elution using a 5 to 90% ACN gradient containing 0.1% trifluoroacetic acid. A reverse phase Dynamax (Palo Alto, CA) C18 column (250 × 41mm) was used and eluted proteins were detected using an ultraviolet (UV) detector set to wavelength 280 nm. The MU1140-containing fraction, determined by bioassay, was collected, diluted with buffer B (acidified water) and again loaded onto the C18 column. Elution was performed with a 0 to 100% methanol gradient containing 0.1% trifluoroacetic acid. The MU1140-containing fraction (52-58 minutes) was again collected and lyophilized to dryness. Lyophilization resulted in separation of MU1140 as white fluffy strands over a dark, crusty pellet. The MU1140 fraction recovered and stored at -20^{o}C until use.

Solubility and Stability Assessment

As MU1140 is intended to be administered as an IV solution, its solubility limit and stability in normal saline were tested. Solubility was tested by addition of various amounts of MU1140 to a fixed amount of normal saline at room temperature. The samples were vortexed and centrifuged at 16,000 ×g for 30 minutes at room temperature. Vials that showed a pellet were regarded to contain MU1140 in a concentration that surpassed MU1140's solubility limits. The stability of MU1140 in saline was tested by incubating the lantibiotic in saline at room temperature and at 4^{o}C for 24 hrs. Aliquots were removed at 1, 2.5, 5, 8, and 24 hrs, and bioassayed for their MU1140 content.

Results and Discussion

To determine cultivation parameters that produce optimal MU1140 production, various cultivation parameters were varied. The amount of MU1140 produced was determined by a deferred antagonism bioassay using *M. luteus*.

Growth Medium

Six different commonly used media (Table 1) were tested to identify a medium best suited for MU1140 production. It was found that 1.25% maltose as the carbon source, 3% yeast extract (YEX) which served as the culture's nitrogen source, and 1.25% $CaCl_2$ proved optimal for MU1140 production. Further medium optimization was achieved by using TSYEX as a base model media, varying the above mentioned components, and the subsequent MU1140 titer was noted. The percentage of yeast extract in the fermentation medium was varied between 2.5– 10% and maltose was varied between 2 – 4%. The increase in MU1140 titer was proportional to the medium's yeast extract concentration up to 5%, after which MU1140 titer plateau (figure 2-2). There was no difference between 2% or 4% maltose in the fermentation medium, but maltose was superior to glucose when

compared in a side-by-side fermentation. Figure 2-3 summarizes MU1140 formation when different carbon sources were used.

Oxygen Tension

To determine whether there is a correlation between the dissolbed oxygen concentration and the observed MU1140 titer, the oxygen content of the fermentation broth was varied from slightly aerobic (2% oxygen saturation) to completely aerobic (100% oxygen saturation). The amount of dissolved oxygen was controlled and maintained at a constant level by varying the stirrer speed and thus medium aeration state. An oxygen-probe was used to measure the medium oxygen content. Figure 2-4 summarizes the MU1140 titer and cell's biomass observed as a function of fermentation medium oxygen tension. The highest MU1140 titer was observed when oxygen was maintained at 2% of maximum saturation. As oxygen tension increases, and although the culture's biomass increases proportionally, the MU1140 titer decreased sharply with the lowest titer observed was at media's oxygen at saturation (100%).

Salts

Production media of other lantibiotics (8) were known to contain the salt $CaCl_2$, thus we investigated the effect of this salt on the production of MU1140. To identify the $CaCl_2$ concentration that is optimal for MU1140 production, different $CaCl_2$ media content was varied from 0 - 2%. $CaCl_2$ did not have any effect on the MU1140 titer. However, divalent cations have been implicated in lantibiotic resistance of the producer strain (13). This could be due to neutralization of the bacterial outer cellular anionic charge by the divalent cations and thus reduced interaction of the cationic lantibiotic to the bacterial cell. At this point the role of calcium is not clear.

pH and Inoculum Size

MU1140 fermentation is carried out using a constant pH that varied from pH 5 to 6 at increments of 0.2 pH units. It appears that the amount of MU1140 produced is highly affected by the pH applied during fermentation. pH 5 - 5.6 resulted in almost the same titer with pH 5.2 inducing the highest MU1140 titer (figure 2-5). MU1140 titer significantly dropped at pH above 5.6 although *S. mutans* biomass did not change but rather plateau. MU1140 production increased as the percentage inoculum volume increased to peak at 10% which corresponds to a titer of 1600, after which the titer drops drastically to < 25 at 50% as well as 100% inoculum size (figure 2-6).

Purification

Before start of the purification process, the presence of MU1140 activity is confirmed in the culture liquor by biasssys (figure 2-7). A typical chromatogram of a run of the MU1140 containing culture liquor after IPA extraction is shown in figure 2-8. Antimicrobial activity was found to be concentrated in a fraction between 25-30 minutes as shown using bioassay (figure 2-9). The material eluted from the column is further diluted and separated on the same C18 column, activity is eluted using an acidified (0.1% TFA) methanol gradient (figure 2-10) which elutes as a single peak in fraction 55-60 minutes. Results are confirmed by bioassay of the mentioned fraction (figure 2-11). Post lyophilization of the methanol eluted material, MU1140 appears as an off beige/ white matter (figure 2-12) with molecular weight 2266 Da. Analytical HPLC the MU1140 shows a single peak and MS analysis shows one major ion with *m/z* of 1133 which corresponds to the doubly charged MU1140 species. The above analysis suggests that according to these methods it appears that MU1140 produced in our laboratory is homogenous.

Solubility and Stability Assessment

Solubility has been estimated to be >100 µg/µl. The stability study of MU1140 in saline shows a 20% drop in MU1140 concentration at both temperatures which occur in the first few minutes followed by a stable and unchanging concentration of MU1140 for 24 hrs.

Conclusion and Discussion

In this work, growth medium components and environmental factors that trigger the production of the lantibiotic MU1140 have been described. It was observed that 5% yeast extract, 0.5% calcium chloride, and 4% glucose yielded the highest amount of MU1140. The purification method consisted of a precipitation step using ammonium sulfate to crash down the bioactivity followed by selective uptake in 80% IPA and two separation steps using reversed phase chromatography where the activity is eluted after the first separation using acetonitrile and after the second separation using methanol. The activity containing fraction is lyophilized and MU1140 is left behind as a fluffy white matter. The yield of MU1140 according to this protocol is less than 1mg/liter which does not enable large scale commercialization of this antibiotic. This low yield could be explained by the susceptibility of the producer strain to the antibiotic. To prevent self-kill, the producer strain could utilize negative feedback loops to down-regulate the MU1140 production and maintain the antibiotic concentration below toxic levels. MU1140 was shown to be very soluble in saline and stable for over 24 hours. Collectively, the data demonstrates that the optimization process has led to an increase in MU1140 formation. Although the yield of MU1140 using our method does not support commercialization of this antibiotic, this work can be used to further our understanding of the complex circuitry that triggers and controls the production of these potent cell killers.

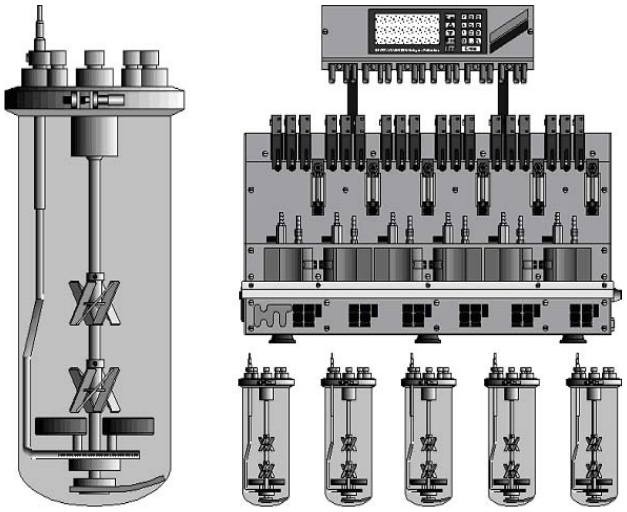


Figure 2-1. The Sixfors Fermentor. A. Illustration of a single vessel. B. The complete sixfors apparatus with six fermentation vessels shown at the bottom.

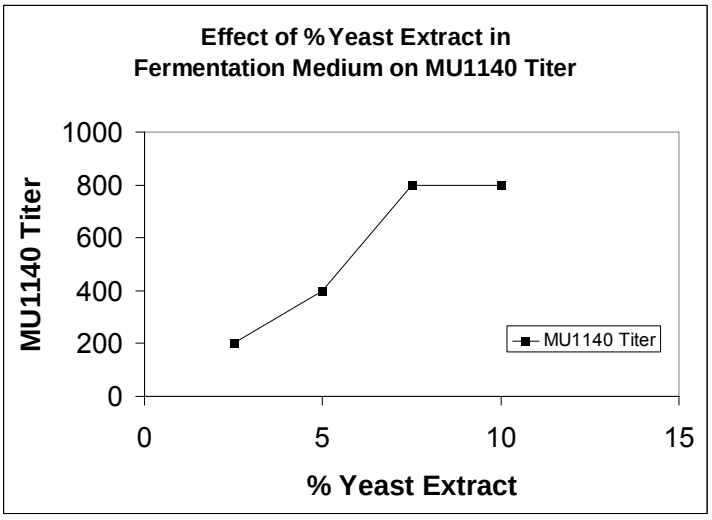


Figure 2-2. Maximum MU1140 production is observed when yeast extract is at concentration equivalent to 7.5% (w/v) of the fermentation medium

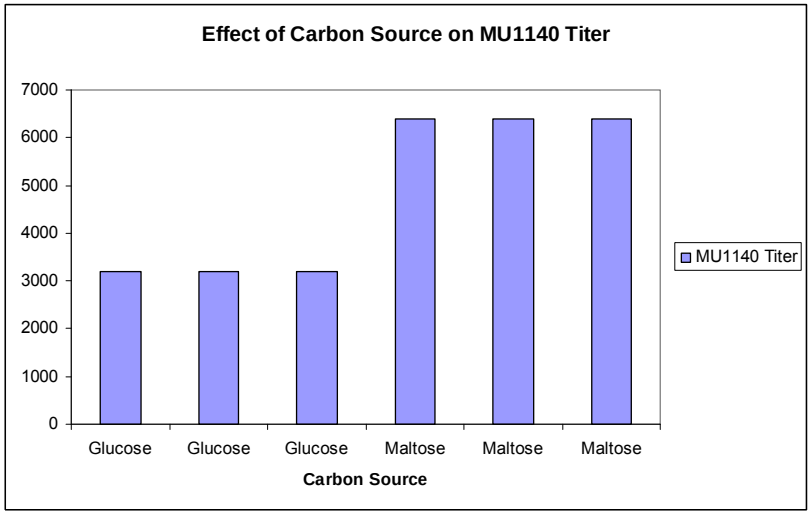


Figure 2-3. Maltose is a better inducer of MU1140 production when compared to glucose. In all Sixfors vessels containing maltose as the sole carbon source in the medium, MU1140 titer was double that observed in vessels containing glucose as the carbon source.

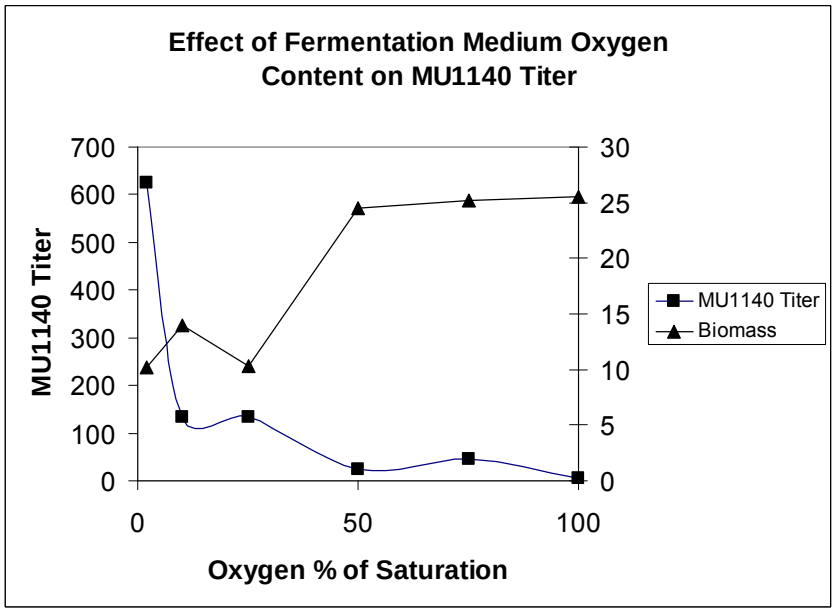


Figure 2-4. Comparison between MU1140 production culture's biomass as a function of Oxygen saturation of fermentation medium.

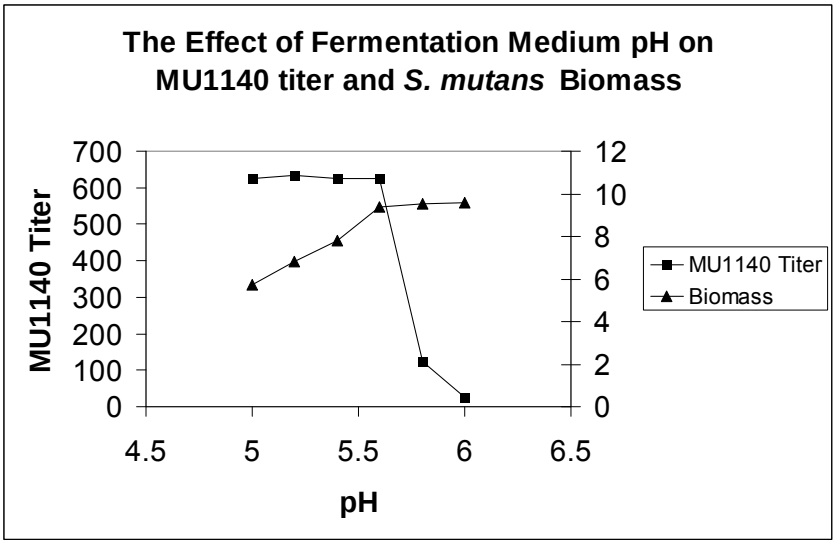


Figure 2-5. The effect of fermentation medium pH on MU1140 production.

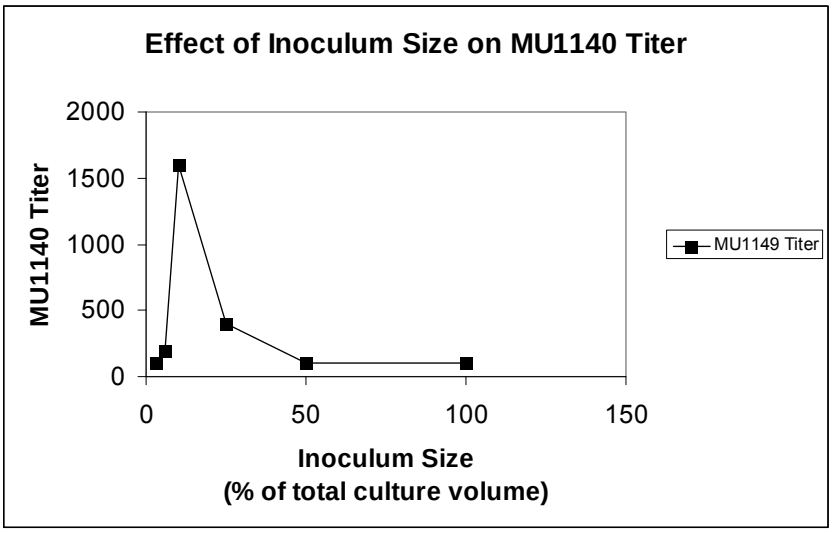


Figure 2-6. MU1140 concentration in the fermentation broth in relation to the inoculum size.

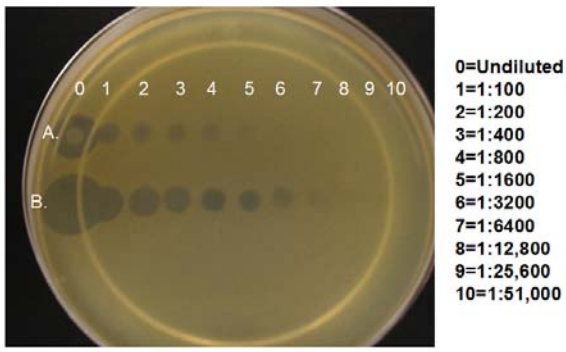


Figure 2-7. Bioassay of fermentation culture liquor containing MU1140. Spots labeled (A) are 1:100 dilutions from the culture liquor followed by 2-fold dilutions. Spots labeled (B) are standard MU1140 solution. This technique is used to monitor and quantify the amount to MU1140 produced per batch.

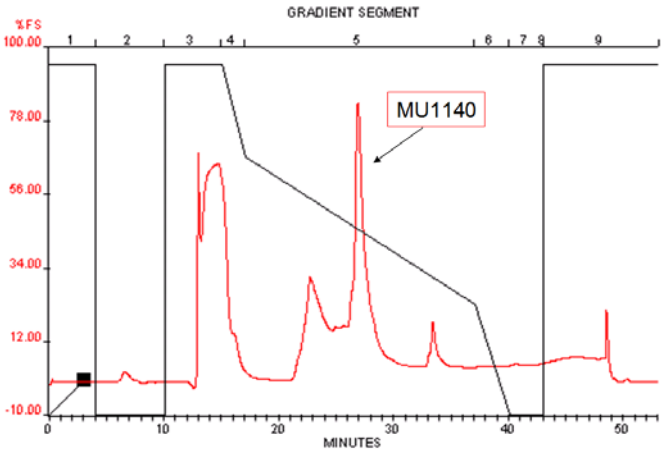


Figure 2-8. Chromatogram of HPLC run of IPA extracted MU1140 from ammonium sulfate precipitation of culture liquor precipitate.

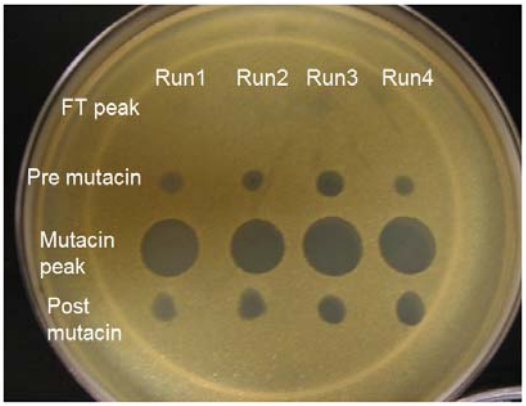


Figure 2-9. Bioassay of eluent fractions of C18 column. The lane labeled Mutacin peak shows the most bioactivity which corresponds to the peak collected in the 26-28 minutes fraction.

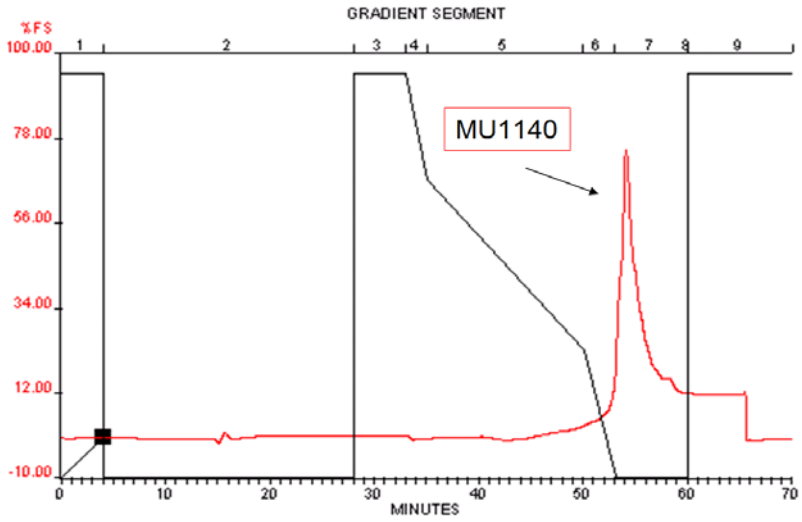


Figure 2-10. Chromatogram of HPLC methanol run. Material eluted off the C18 column using ACN diluted and rechromatographed again on the C18. MU1140 eluted using an acidified methanol gradient.

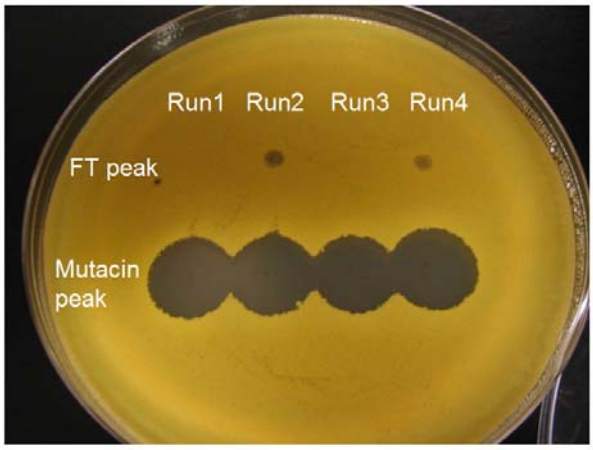


Figure 2-11. Bioassay of eluent fractions of C18 column. The lane labeled Mutacin peak shows the most bioactivity which corresponds to the peak collected in the 52-58 minutes fraction.

Figure 2-12. Purification by lyophilization. The material eluted from the C18 column using acidified methanol lyophilized. A) Fluffy white MU1140. B) Crusty pellet containing contaminants.

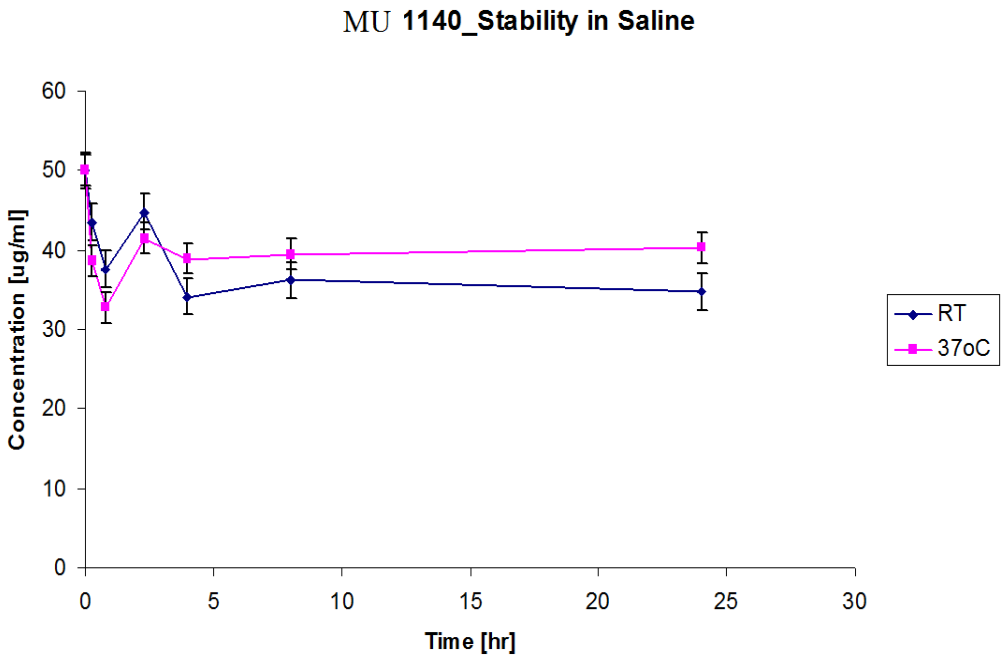


Figure 2-13. Stability of MU1140 in saline. An initial drop in concentration is observed followed by no change in concentration.

Tables

Table 2-1. Comparison of MU1140 concentrations obtained with different commercial media.

Media*	MU1140 Titer
Media 1: 1% YEX, 0.5% $CaCl_2$, 4% Maltose	1000
Media 2: THB, 0.5% $CaCl_2$, 4% Maltose	<25
Media 3: TSB, 0.5% $CaCl_2$, 4% Maltose	<25
Media 4: LB, 0.5% $CaCl_2$, 4% Maltose	<25
Media 5: BHI, 0.5% $CaCl_2$, 4% Maltose	<25
Media 6: THB, 0.3%/YEX, 4% Maltose	<25

* YEX (yeast extract), THB (Todd Hewitt Broth), TSB (Tryptic Soy Broth), LB (Lauriat Broth), BHI (Brain Heart Infusion).

CHAPTER 3
DEVELOPMENT AND VALIDATION OF AN EXTRACTION AND LC/MS QUANTIFICATION METHOD FOR THE LANTIBIOTIC MU1140 IN RAT PLASMA

This study reports the first ever development and validation of an extraction and quantification method for a lantibiotic in plasma. This method was developed for the quantification of total MU1140 in Sprague Dawley rat plasma. The procedure involved acidification of plasma samples with formic acid followed by precipitation of plasma proteins using isopropanol. The samples were analyzed by RPLC/MS. Gallidermin was used as an internal standard (ISTD). The analyte and ISTD were eluted using a gradient of isopropanol and water, both acidified with 0.3% formic acid (v/v), at a flow rate of 250µl/min. Positive electrospray ionization was utilized at the ion source and the analyte and ISTD were both detected by selected-ion monitoring (SIM). Total run time was 15 minutes. This method was validated for selectivity, sensitivity, linearity, recovery, accuracy, and precision. The method was shown to be selective, with a quantitative linear range of 0.39 – 100 µg/ml using 25 µl samples. The mean extraction recovery for MU1140 was 96% ± 0.4%. The bias, intra- and inter-day percent relative standard deviation at all concentrations tested was lower than 15%. The analyte was shown to be stable to freeze/thaw and for short and long term storage. Extracted MU1140 was stable at 4^{o}C for over 5 days.

This method was successfully applied to a preliminary pharmacokinetic study of intravenously administered MU1140 in Sprague Dawley rats. Overall, this method is shown to be applicable for quantification of MU1140 in plasma samples for the purpose of further MU1140 ADME or bioequivalence studies.

Introduction

The excessive and improper use of antibiotics has lead to the selection and spread of bacterial strains resistant to many of the currently used antibiotics. The United States FDA noted that antibiotic resistance problems must be detected as they emerge, and actions taken to contain them, or else the world could be faced with previously treatable diseases that have again become untreatable, as in the days before antibiotics were developed (40). This sharp increase in bacterial antibiotic resistance can be contained by the development and commercialization of new classes of antimicrobials (75).

MU1140 (Figure 3-1A) is a bacteriocin produced by the microorganism *Streptococcus mutans* strain JH1140 (37). It belongs to the family of antimicrobial peptides known as lantibiotics, so named for their content of lanthionine residues (79). Lanthionines are amino acids that are composed of two alanine residues linked by a thioether bridge through their β-carbons. MU1140 has been shown to exert its antimicrobial effect on Gram positive bacteria by a novel mechanism involving lipid II abduction, in which aggregates of MU1140 bind to molecules of lipid II and translocate them from sites of active cell wall biosynthesis (35). The result is inhibition of cell wall synthesis.

Although the first lantibiotic was discovered in 1928 (71), and approximately 50 more have been identified subsequently, their development as pharmaceutical agents for treatment of infectious diseases has been hindered by the lack of cost effective production and/or purification (15). In the case of MU1140, production of sufficient amounts of essentially pure product has been achieved in order to perform a number of pre-clinical tests (29). These indicate the potential usefulness of MU1140 in the treatment of certain

Gram positive infections, including those caused by wild-type and drug resistant variants of *Staphylococcus aureus, Streptococcus pneumoniae,* and *Enterococcus faecalis*.

A reliable bioanalytical method for the quantification of drugs is crucial for their development (61). Determination of the drug's concentration is needed for many reasons, among which is the assessment of the pharmacokinetic properties of the drug and the subsequent dose design, evaluation of stability, assessment of patient compliance, therapeutic drug monitoring, and determination of the bioequivalence of generics and follow on biologics. To date, no analytical method has been reported for the quantification of free or total lantibiotics in a biological matrix. This study describes the development and validation of an LC/MS method for the quantification of total MU1140 in rat plasma. The method was validated with regard to its accuracy, precision, selectivity, sensitivity, reproducibility, and stability.

Experimental

Materials and Stock Solutions

MU1140 was produced by Oragenics, Inc. (Alachua, FL) and gallidermin (Figure 3-1B), which was used as an internal standard (ISTD), was purchased from Alexis Biochemicals (San Diego, CA). MU1140 and the gallidermin ISTD stock solutions were prepared in 1:1 (v/v) mixture of isopropyl alcohol (IPA):water at a concentration of 25 µg/ml and stored at -80^{o}C until used. Mass spectrometry grade IPA, water, and formic acid were purchased from Sigma (St Louis, Mo). Microcon® Centrifugal Devices (10 KDa cutoff) were purchased from Millipore (Bedford, MA). Drug-free, male Sprague Dawley rat plasma with EDTA was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA) and aliquoted and stored at −20^{o}C until used.

Equipment and Analysis Conditions

The LC/MS analysis system used consisted of a Surveyor plus autosampler and pump (ThermoFisher Scientific, San Jose, CA) coupled to an API SCIEX 150EX single quadrupole mass spectrometer (Concord, ON, Canada) equipped with electrospray ionization. A Clipeus C-18 analytical column (100x2.1 mm; 5 µm particle size; Higgins, MA, USA) with a pre-column in-line filter (0.5µm, MacMod, PA) was used for separation at room temperature. Samples (25 µl) of standards and unknowns were injected onto the column. Proteins were eluted with an acidified (0.3% v/v formic acid) IPA:water gradient at a flow rate of 250 µl/min. The gradient went from 5% to 95% IPA:water (v/v). Electrospray ionization was used for ions generation, with positive ion detection. Optimal sensitivity was achieved when ion source temperature was maintained at 475°C and a voltage of 5.5kV was applied to the sprayer needle. Nitrogen was used as the nebulizer and curtain gas. Single ion monitoring (SIM) was used for detection of analyte and ISTD. SCIEX Analyst software 1.4 was used for data collection and integration of the chromatographic peaks. The peak area ratios of MU1140 to ISTD were plotted as a function of MU1140 concentration in standard solutions. A linear curve fit with no weighing was used to generate the regression line. The regression equation of the calibration curve was used to calculate the concentrations of the quality control samples and all unknowns.

Standards and Quality Control Samples

Working solutions of MU1140 (1 µg/µl) and gallidermin (0.1 µg/µl) were prepared in 10% IPA. These solutions were used to prepare calibration curve standards and QC samples. Calibration standards of MU1140 in rat plasma were prepared by addition of MU1140 working solution to an initial concentration of 100 µg/ml and nine serial 2-fold

dilutions were prepared. Quality control (QC) samples were prepared at 3 concentrations, including low (1 µg/ml, LQC) medium (10 µg/ml, MQC), and high (50 µg/ml, HQC). Both the calibration standards and quality control samples were spiked with the working solution of the ISTD to a final concentration of 6 µg/ml.

Sample Preparation

Plasma samples were spiked with the ISTD working solution to give a final concentration of 6 µg/ml. Samples from the pharmacokinetic study were allowed to thaw unassisted and fortified with the ISTD for a final concentration of 6 µg/ml. The samples were mixed for 30 seconds at medium speed using a vortex (VWR, Chicago, IL, USA). MU1140 and ISTD were extracted by acidifying the samples by addition of 100% formic acid to a final concentration of 2% (v/v) and vortexing for 30 seconds. Each sample was diluted with an equal volume of 100% IPA and vortexed for 10 seconds to ensure complete mixing, after which the samples were centrifuged at 16,000 x *g* for 30 minutes in a table top centrifuge (Eppendorf, Hamburg, Germany) at room temperature. The supernatant was transferred to Microcon ultrafiltration device and centrifuged to dryness at 10,000×*g* at room temperature. The ultrafiltrate was analyzed by LC/MS as described above.

Method Validation

The method was validated for selectivity (specificity), sensitivity, linearity, accuracy, precision, recovery, and stability. The selectivity of this method was verified by treating blank rat plasma samples from six different lots and analyzing the samples for interfering peaks with the same *m/z* ratio at the analyte and ISTD retention times.

Sensitivity was assessed by determining the lowest quantifiable concentration (LLOQ) of MU1140. The LLOQ was established as the lowest concentration of

MU1140 used in the calibration curve with accuracy and precision of 100% ±20%. Bias and relative standard deviation were used as measures of accuracy and precision respectively, and were computed using

$$\text{Bias} = \frac{\text{Theoretical Concentration} - \text{Mean Observed Concentration}}{\text{Theoretical Concentration}} \times 100$$ and % Relative

$$\text{Standard Deviation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$. Linearity was assessed by plotting MU1140:ISTD peak area ratios *versus* concentrations of calibration curve standards.

Accuracy and precision were assessed by injecting QC samples in pentuplicate and quantifying the MU1140 concentration using the regression line equation of the calibration curve. Bias and relative standard deviation were used as measures of accuracy and precision, respectively, and calculated as mentioned above. A run was rejected if more than a third of the QC sample concentrations showed a deviation from the theoretical concentration equal to or greater than 20%.

The developed method's ability to recover MU1140 was estimated by quantifying the MU1140 content of QC samples extracted by the developed method using a calibration curve constructed from unextracted standards. Unextracted standards were prepared by fortifying extracted, drug-free plasma filtrate with MU1140 and ISTD. These samples represent 100% recovery and normalize for matrix effect, if any.

Stability of MU1140 under different conditions was assessed as part of the method's validation procedure. MU1140 stock solution stability was assessed at -80^{o}C for up to 30 days. Every 10 days, 3 aliquots were thawed, spiked with ISTD, extracted, and analyzed. Bench top (short term) stability was determined at three concentrations (LQC, MQC, and HQC). Plasma aliquots were fortified with MU1140 and incubated at

room temperature for 1.5, 3, and 6 hours. After incubation, the ISTD was added to the samples and the samples were extracted and analyzed for their MU1140 content. Freeze and thaw stability was evaluated by subjecting rat plasma samples spiked with MU1140 at three different concentrations (2.5, 10, and 40 μg/ml) to three freeze-thaw cycles. Samples were frozen for 24 hours at -80^{o}C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle, samples were spiked with ISTD, extracted, and analyzed. To determine the post-preparative stability of MU1140, plasma samples were spiked with the MU1140, samples were extracted as per the developed method and incubated at 4^{o}C for up to 4 days. ISTD was added to the samples prior to analysis.

Preliminary Pharmacokinetic Study

MU1140 doses equivalent to 12.5 mg/kg or 25 mg/kg rat body weight were administered via the indwelling jugular cannula to two rats as a rapid iv infusion (< 1 minute) and plasma samples were drawn via the cannula at 5, 10, 20, 30 minutes, and 1, 2, 4, and 6 hours post dosing in anticoagulant containing tubes. Blood samples were centrifuged at 500 ×g for 10 minutes to separate the plasma. Plasma samples were immediately collected and stored at -80°C until analyzed. The validated method was used to quantify the rat plasma samples' MU1140 content.

Results and Discussion

LC/MS Detection and Method Selectivity

Analysis conditions for LC/MS were optimized using MU1140 and gallidermin (ISTD) in 50% IPA. The run time of the chromatographic method was 15 minutes with retention times of the analyte and ISTD being approximately 5.2 and 5.3 minutes respectively. These methods were used in the following studies. Chromatograms of rat

plasma spiked with MU1140 and ISTD and then extracted revealed that each molecular species was dominant in its doubly protonated molecular ion form $(M+2H)^{2+}$, detected at *m/z* of 1133 and 1083, respectively. These values accord with their known molecular formulas. Six different lots of drug-free rat plasma were treated as per the developed method and analyzed by LC/MS. No endogenous matrix ions were observed at *m/z* 1133 or 1083 at the retention times of MU1140 and ISTD. Data are presented in Figures 3-2 and 3-3. This ensured the selectivity of the method and its applicability to quantify these lantibiotics in rat plasma.

The relatively short run time allowed increased sample throughput, thus making this method specifically suitable for quantitation needs of studies of large sample size such as pharmacokinetic or bioequivalence studies. To increase sensitivity, selective ion monitoring (SIM) was used for quantification. Gallidermin was found to be a suitable internal standard due to its structural similarity to MU1140.

Linearity and Sensitivity

The calibration curve was linear over the range of 0.39 – 100 µg/ml when 25µl of sample was injected onto the column. The relatively small sample volume allowed multiple injections from the same sample, thereby improving precision of quantitation. The correlation coefficient (r^2) was > 0.995 for all validation batches.

The limit of quantification for MU1140 was far below the established MIC of MU1140 for susceptible organisms (Ghobrial et al, *submitted*). This finding suggests that accurate quantification of MU1140 in the concentration range of interest should be readily achieved, and lead to accurate determination of the pharmacokinetic and pharmacodynamic parameters, and robust PK/PD modeling.

Accuracy, Precision, and Recovery

The inter-day accuracy and precision of the method were determined at the LLOQ as well as at three different QC concentrations in two different days. The accuracy of the method was described by the bias of theoretical versus measured concentrations, while the percentage of the relative standard deviation (% RSD) served as a measure of precision. Table 1 summarizes the intra-run, as well as the inter-day accuracy and precision of this bioanalytical method, measured on two different days. The intra-run bias was < 11% for all concentrations with intra-run %RSD of < 16 at the LLOQ level, and < 11% for all other concentrations. The mean inter-day deviation from the nominal concentration was < 8% and the inter-day RSD was < 2% for all tested concentrations. These data are in compliance with FDA guidance on bioanalytical method validation (82).

The developed method's percentage recovery of MU1140 from the plasma samples was estimated by comparing the ratio of the analyte peak areas from extracted samples to that from the unextracted samples. The mean recovery of MU1140 for all samples was 96.05% with RSD of < 11%. Data are summarized in Table 2.

The data presented above confirms that the developed method is capable of accurately and precisely quantifying MU1140 in Sprague Dawley plasma. This high recovery ratio improves the ability to detect and quantify MU1140.

Stability

Stability of the MU1140 stock solution constituted at 25µg/ml in 50% IPA /water (v/v) was assessed after freezing for 10, 20, and 30 days at -80°C. The original solution was aliquoted into twelve tubes. Three tubes were analyzed immediately and the others were frozen at -80^{o}C. Three tubes were thawed and analyzed at the indicated times.

Each vial was sampled in triplicate. In all cases, MU1140 was detectable at levels equal to or greater than 96% (RSD < 8 %) compared to MU1140 freshly prepared at the same concentration. Data are presented in Table 3. This suggests that the standard solution of MU1140 was stable for at least 30 days when stored at -80°C.

Bench top stability at room temperature of MU1140 in plasma was investigated at the three QC concentration levels, LQC, MQC, and HQC. Just before termination of the incubation period the ISTD was added. Incubation at room temperature was stopped at 0, 1.5, 3, and 6 hrs by addition of formic acid and isopropanol as per the extraction procedure and samples were analyzed for MU1140 content. There was no measurable loss of MU1140 in plasma at room temperature for more than 6 hrs. Data are summarized in Table 4.

Plasma samples spiked with MU1140 were subjected to three freeze and thaw cycles, after which ISTD was added, and the samples were processed by extraction and quantification. A mean percentage change of < 0.5% with a % RSD of < 10% was observed. Data are summarized in Table 5. This result confirms that multiple freezing and thawing of MU1140-containing plasma did not affect the stability of MU1140.

Post-preparative stability of MU1140 was also determined at three concentrations at 4°C for up to 4 days. Less than 5% (< 12 % RSD) change in the intensity of the MU1140 signal was evident. Data are summarized in Table 6. This result indicates that MU1140 extracted from plasma was stable for at least 4 days at 4°C.

Optimal storage and handling condition of MU1140-containing plasma samples were tested by the long-term and short-term stability studies, multiple freeze/thaw studies, and post-preparative stability studies which all showed no appreciable

degradation and loss of the lantibiotic. These findings indicate that plasma samples from dosed animals can be conveniently stored at -80°C and thawed, processed on the bench top at room temperature, and placed in a refrigerated autosampler for extended periods without significant loss of MU1140.

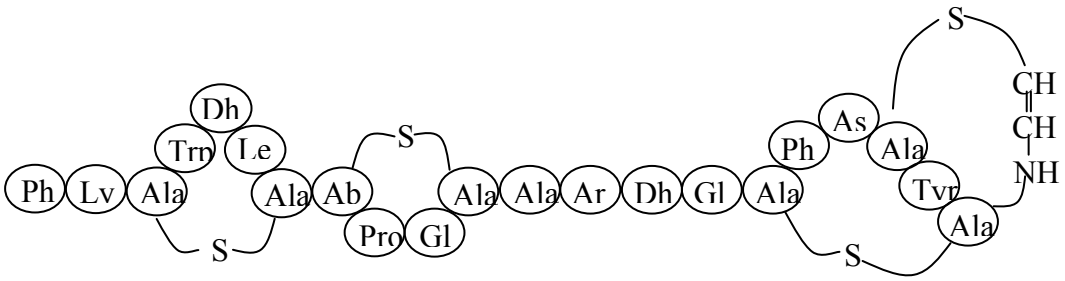
Preliminary Pharmacokinetic (PK) Study of MU1140 in Sprague Dawley Rat

The purpose of this PK study was to evaluate the validated method for the quantification of MU1140 content of in vivo samples. The method was successfully applied to a preliminary pharmacokinetic study of MU1140 in two rats which received either 12.5 or 25 mg/kg dose. All samples were analyzed within 1 day and precision and accuracy for QC samples were within acceptable limits. Plasma concentration-time data were subjected to noncompartmental analysis (NCA) and dose linearity of the calculated pharmacokinetic parameters was established. Cmax and AUC0-∞ were dose dependent and measured to be 8.86, 15.9 µg/ml and 12.39, 24.69 hr. µg/ml for the 12.5, 25mg/kg doses, respectively. The half life and clearance were dose independent indicating the linearity of the pharmacokinetics of MU1140 in that dose range. The pharmacokinetic profiles are presented in Figure 3-4 and the pharmacokinetic data are presented in table 3-6. Overall, it appears that the developed method is reliable for the in vivo of quantification of MU1140.

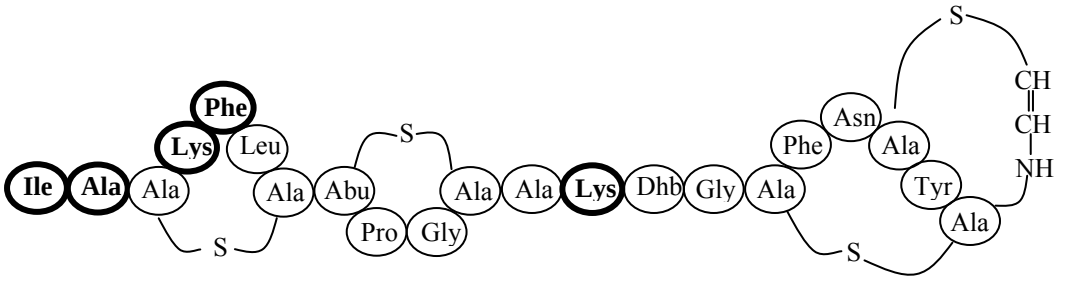
Conclusions

This paper describes the first bioanalytical method for the quantification of a lantibiotic in a biological matrix. The method was developed specifically for the quantification of MU1140 in rat plasma samples, and uses a simple and inexpensive liquid–liquid extraction followed by a rapid, sensitive LC/MS separation and detection procedure. This method was validated to be selective, accurate, precise, and sensitive,

and the stability of MU1140 was not compromised during sample handling and processing. The method was tested for the quantification of MU1140 in rats following IV administration. Clear dose-dependent response was observed, ensuring the validity of this quantification method. Collectively, this method should be applicable to all quantitative studies of MU1140 development and should serve as a starting point for optimization of bioanalytical methods for other lantibiotics.



A



B

Figure 3-1. Analyte and ISTD. A) MU1140 (37) B) Gallidermin (42). Amino acids different from MU1140 are highlighted.

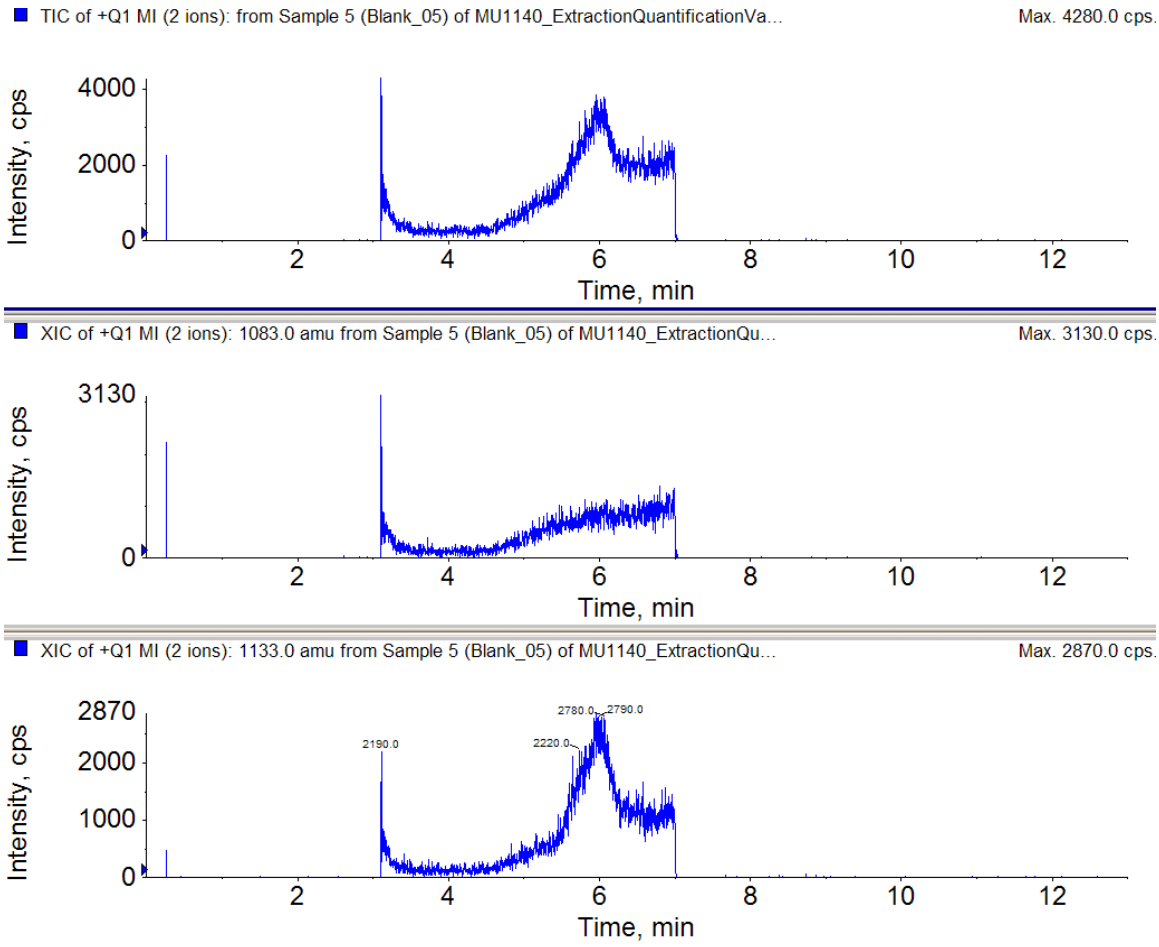


Figure 3-2. Representative LC/MS chromatogram of extracted drug-free rat plasma A. Total ion current (TIC) of the two ions B. Extracted ion chromatographs (XIC) for *m/z* 1082-1084 (gallidermin) C. XIC for *m/z* of 1132-1134 (MU1140).

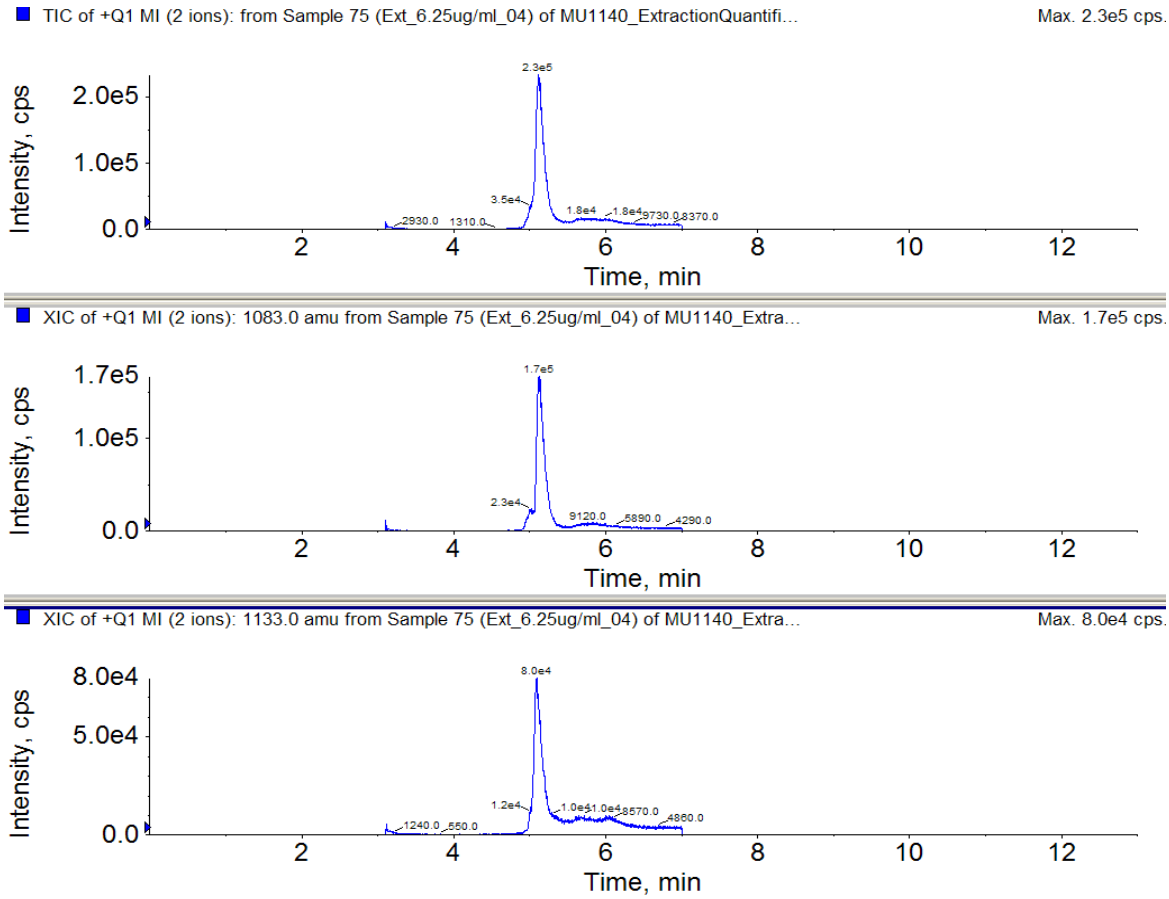


Figure 3-3. Representative LC/MS chromatogram of plasma sample fortified with MU1140 (at LLOQ), *m/z* 1133 and gallidermin, *m/z* 1083. A. TIC of the two ions B. XIC for *m/z* 1082-1084. C. XIC for *m/z* of 1132-1134.

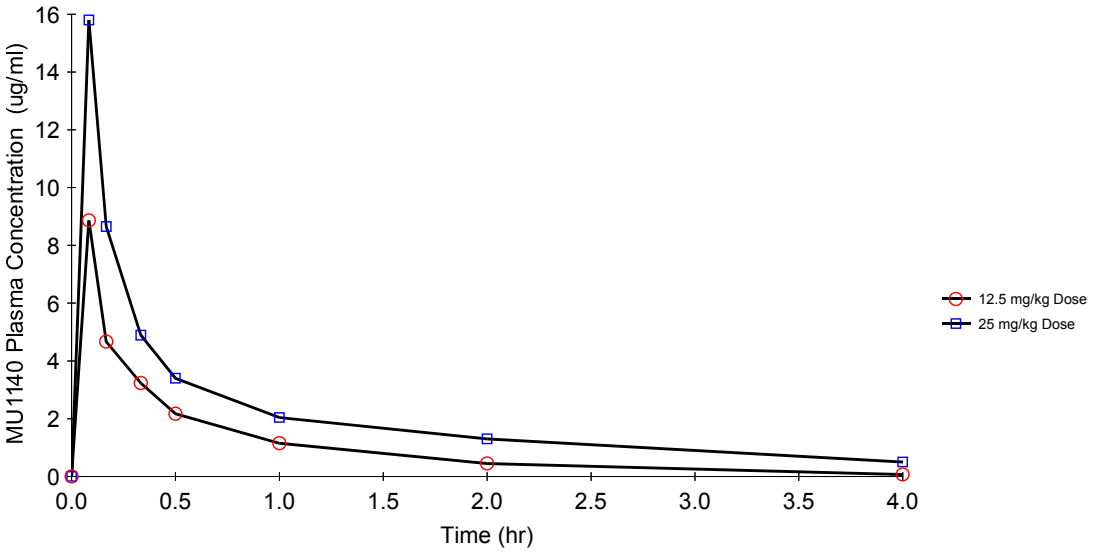


Figure 3-4. MU1140 plasma concentration-time profiles after IV bolus administration of a single dose of 12.5 mg/kg or 25 mg/kg to two different rats.

Table 3-1. Intra-run and inter-day accuracy and precision of the bioanalytical method at the LLOQ and three concentrations of MU1140.

Day 1

	Theoretical Concentrations			
	LLOQ (0.39µg/ml)	1µg/ml	10µg/ml	50µg/ml
	Measured Concentrations (µg/ml)			
Within Run Mean (n=5)	0.36	1.07	9.64	49.39
% Bias	-6.67	6.86	-3.58	-1.23
% RSD	10.7	11.87	8.47	5.1

Day 2

	Theoretical Concentrations			
	LLOQ (0.39µg/ml)	1µg/ml	10µg/ml	50µg/ml
	Measured Concentrations (µg/ml)			
Within Run Mean (n=5)	0.35	1.10	9.85	48.9
% Bias	-10.26	10.0	-1.50	-2.20
% RSD	15.7	7.92	6.69	4.68

Inter-day Comparisons

	Theoretical Concentrations			
	LLOQ (0.39µg/ml)	1µg/ml	10µg/ml	50µg/ml
Inter-day Mean (n=2) Concentration	0.36	1.09	9.70	49.2
% Bias	-8.97	8.50	-2.55	-2.14
% RSD	1.99	1.95	1.52	0.70

Table 3-2. Recovery of MU1140 from plasma samples.

Theoretical Concentration (μg/ml)	2.5	10	40
Mean (n=6) Observed Concentration (μg/ml)	2.39	9.64	38.46
% RSD	10.7	9.1	6.4
Recovery (%)	95.6	96.4	96.15

Table 3-3. MU1140 stock solution (25 μg/ml) stability at –80°C for up to 30 days.

Day	Mean (n=3) Concentration (μg/ml)	% Bias	% RSD
0	24.94	0.24	6.38
10	24.09	3.64	7.65
20	25.73	-2.92	6.4
30	25.13	-0.52	7.89

Table 3-4. Bench top stability of MU1140 in Sprague Dawley plasma at room temperature. % RSD is shown in parenthesis.

	Theoretical Concentration (μg/ml)		
Time (hr)	1	10	50
	Calculated Concentration (μg/ml)		
0	0.98 (9.5)	10.2 (7.9)	51.1 (4.7)
1.5	1.10 (10.6)	9.95 (8.2)	49.9 (4.5)
3	1.20 (11.3)	10.3 (8.6)	51.6 (3.3)
6	0.95 (8.8)	9.50 (10.5)	50.3 (5.5)

Table 3-5. Freeze/thaw stability assessment of MU1140 in plasma.

Theoretical concentration (μg/ml)	Mean (n=6) Concentration (μg/ml)	% Bias	% RSD
2.5	2.29	0.06	9.1
10	10.0	0.36	8.4
40	42.5	0.49	6.3

Table 3-6. Post-preparative stability assessment of MU1140 after 4 days at 4°C in autosampler.

Day	Theoretical Concentration (µg/ml)		
	2.5	10	40
	Mean (n=3) Concentration (µg/ml)		
0	2.30 (11.3)	8.64 (8.1)	38.9 (5.3)
1	2.40 (9.6)	9.30 (10.2)	37.2 (6.2)
2	2.30 (9.2)	10.2 (9.7)	37.7 (5.8)
3	2.55 (9.8)	10.0 (8.7)	40.5 (7.4)
4	2.45 (10.3)	10.1 (9.1)	36.5 (6.1)
% Bias	-3.00	-3.37	-4.60

Table 3-7. MU1140 pharmacokinetic parameters determined using NCA analysis of plasma concentration-time data.

	Pharmacokinetic Parameters			
Dose	C_{max} (µg/ml)	$AUC_{0-\infty}$ (hr. µg/ml)	$t_{½.}$ (hours)	Clearance (l/hr/kg)
12.5 mg/kg	8.86	12.39	1.33	1.00
25 mg/kg	15.9	24.69	1.56	1.01

CHAPTER 4
PHARMACOKINETIC/PHARMACODYNAMIC EVALUTATION OF THE LANTIBIOTIC MU1140 IN SPRAGUE DAWLEY RATS

This is the first study to report the pharmacokinetics (PK) of a lantibiotic in an animal model and a linked *in vivo* pharmacokinetic/ *in vitro* pharmacodynamic (PK/PD) model to predict its *in vivo* activity. This work was done using the lantibiotic MU1140, an antibiotic in preclinical development that is indicated for the management of Gram positive infections. Following intravenous administration of MU1140 at 25 mg/kg rat body weight, the plasma concentration-time profile of MU1140 in rats declined biexponentially with a mean elimination half-life of 1.7 ± 0.1 hours and a mean maximum concentration of 18.7 ± 5.5 µg/ml. The mean volume of distribution and systemic clearance, calculated from the noncompartmental analysis of MU1140 plasma concentration-time profiles, were 3.48 ± 1.14 L/kg and 1.44 ± 0.42 L/hr/kg, respectively, and the mean total area under the plasma concentration-time curve was 18.7 ± 5.5 µg.hr/ml. Plasma concentrations of MU1140 were measurable up to 6 hours post-administration. The best fit of plasma concentration-time data was achieved using an open two-compartment model with elimination from the central compartment. It was observed that rapid injection of this lantibiotic is associated with a histamine release/hypersensitivity reaction similar to vancomycin's "red man syndrome". Pre-medication with diphenhydramine blocked this response. Using time-kill data of MU1140 *vs. Staphylococcus aureus* (*S. aureus*), a pharmacodynamic (PD) model was developed to explain the observed time-kill profile. The PD model was based on the two subpopulation (susceptible and resistant) strategy. The utility of the developed *in vitro* PD model was further extended by incorporation of the *in vivo* PK model of MU1140 in rats as an input function to yield the perspective linked PK/PD model. This model was

used to simulate *in vivo* time-kill behavior of MU1140 against *S. aureus* in rat. The developed PD model was predictive of the *in vitro* time-kill data and valuable MU1140 PD parameters were estimated, namely the concentration of MU1140 that produced half the maximum kill for susceptible and resistant bacteria (EC_{50S} and EC_{50R}) were calculated to be 0.0001 and 2 μg/ml respectively. Collectively, these findings suggested that by using simulation the developed PK/PD model can be used to study the effect of variation of dose, bioavailability, and dosing regimen on the outcome of therapy and to optimize the dosing regimen for MU1140 and other lantibiotics with similar properties.

Introduction

Soon after they are approved and are in widespread human use, some broad spectrum and regularly prescribed antibiotics lost their ability to control certain bacterial infections(46), and currently more than 70% of nosocomial infections are caused by drug resistant bacteria(12, 41). Annually, infections caused by *S. aureus* alone, which represent 16% of infections nationwide, results in 12,000 inpatient deaths, 2.7 million days in excess length of stay, and 9.5 billion dollars in extra hospital charges(60). Two factors contribute and further exacerbated this antibiotic resistance healthcare crisis. The first is the diminishing antibiotics pipeline(74) especially those with novel mechanisms of action. According to Rice *et al*, since 1998, only ten antibiotics have been approved, out of which only two out possess a novel mechanism of action. The other factor is the continuous and rapid emergence and spread of new bacterial strains that are resistant to currently used antibiotics(70). Thus, there is a need for new antibiotics and appropriate dose and dosage regimen design are crucial to prevent the emergence and spread of bacterial strains resistant to newly developed antibiotics (73).

MU1140 (Figure 4-1) is a lantibiotic produced by the indigenous oral microorganism *Streptococcus mutans* strain JH1140 (37). Lantibiotics (lanthionine-containing antibiotics) are ribosomally synthesized peptides containing various modified amino acids such as lanthionine (Lan, ala-S-ala), methyllanthionine (MeLan, abu-S-ala), didehydroalanine (Dha) and didehydrobuterine (Dhb) (13). These antimicrobial peptides are thought to be excreted in order to eliminate microorganisms competing with the producer microorganism for its habitat. MU1140 has a novel mechanism of action known as "lipid II abduction", which involves inhibition of peptidoglycan cell wall synthesis by binding to and sequestering lipid II(35, 78) away from its site of action, thus, destabilizing the bacterial cell wall resulting in cell lysis. Currently, MU1140 is in the preclinical phase of development as an antibiotic for the management of Gram positive infections.

The purpose of this study was to characterize the plasma concentration-time profile and systemic exposure of a single dose of intravenously administered MU1140 in Sprague Dawley rats. The plasma concentration-time profile will be used to generate a PK model that is predictive of MU1140 concentrations produced by different dosing regimen. Previous time-kill studies show that MU1140 was bactericidal against *S. aureus* when tested *in vitro* (29). By assembling a pharmacodynamic model using time-kill data, very important pathogen and antibiotic specific parameters can be elucidated. Such parameters as the pathogen's growth rate constant (g), the antibiotic's maximum kill effect (K_{max}) and the concentration that produce 50% of K_{max} (EC_{50})(55). To further improve the utility of the PD model, integration of the PK data of the antibiotic with the PD model will create a powerful tool (the linked PK/PD model) that is capable of

predicting the results of therapy in an animal model of infection and can be further modified to design a rational and efficacious clinical dosing regimen that reduces toxicity and the chance of resistance development to MU1140.

Materials and Methods

Drug and Dose Administration

MU1140 was produced by Oragenics, Inc. and was administered over a one-minute period via an indwelling jugular catheter as a single intravenous dose with a volume of administration of 5ml/kg.

Animals

Jugular vein cannulated Sprague Dawley male rats (200-220 g) were purchased from Charles River Laboratories (Raleigh, NC). Animals were housed and allowed to acclimatize for 5 days prior to experiment start day. Experiments were conducted at the University of Florida rodent facility (Alachua, Fl) where they were given Harlan rat chow (7912) and water *ad libitum*. Rats rooms were temperature and humidity controlled.

Experimental Design

All rats were weighed immediately before initiation of the study and the weights were recorded and used for dose calculations. One hour prior to dosing with MU1140, all animals were injected with diphenhydramine (DPA, 20mg/kg) subcutaneously. The jugular cannulae were used to draw the blood samples at times 5, 10, 20, 30 min, and 1, 2, 4, and 6 hrs post-dosing. Blood was mixed 10:1 with a 10× anticoagulant stock solution (15 mg/ml sodium EDTA plus 17 mg/ml sodium chloride) and centrifuged at 500 gs for 20 min at room temperature to separate the plasma. Plasma samples were immediately collected and stored at – 80 $^{\circ}$ C. Normal saline (1 ml) was used to cleanse

the cannulae after MU1140 administration and after blood sampling. Hepranized saline (1000 IU/ml) was used as the cannula lock solution.

Quantification of MU1140 in rat blood samples was carried out using the validated LC/MS method developed by Ghobrial *et al* (28). Briefly, prior to analysis, plasma samples were allowed to thaw at room temperature. Gallidermin, the internal standard, was added prior to sample preparation at a concentration of 6 µg/ml. The sample preparation procedure involved addition of formic acid to a final concentration of 2% (v/v) and vortexing for 30 seconds. Plasma protein precipitation was achieved by addition of an equal volume of isopropanol, followed by centrifugation at 16,000xg for 30 minutes at room temperature. The supernatant was filtered through a 10 KDa MWCO Microcon filter (Millipore, Bedford, MA). The ultrafiltrate was analyzed for its MU1140 content by LC-MS. MU1140 and ISTD were detected by an API 100 single quadrupole mass spectrometer (Applied Biosystems, Concord, ON, Canada) operated in the positive mode with electrospray ionization. Single ion monitoring was used to improve the assay's sensitivity. Quantitation was achieved by monitoring ions at *m/z* 1133 (MU1140) and *m/z* 1083 (gallidermin). The standard curve was linear ($r^2 > 0.998$) and ranged from 0.039 to 100 µg/mL, representing the lower and upper limits of quantitation (LLQ and ULQ), respectively. The relative standard deviation (RSD) for within-run precision was < 15%. Bias was < 7% of the nominal values.

PK Data Analysis

Noncompartmental Analysis (NCA)

WinNonlin (Pharsight Corporation, Mountain View, California) was used to perform the NCA to calculate the area under the concentration-time curve (AUC), total clearance (CL), half-life ($t_{½}$), volumes of distribution (V_{ss} and V_{area}), and the maximum

concentration (C_{max}). The trapezoidal rule was used to estimate $AUC_{0\text{-}tlast}$. The $AUC_{tlast\text{-}\infty}$ (the AUC from the last measured time point to infinity) was determined by dividing the last measured concentration by the elimination rate constant of the terminal phase. The total AUC was the sum of $AUC_{0\text{-}tlast}$ and $AUC_{tlast\text{-}\infty}$. The clearance was calculated as CL = Dose/AUC. The slope of the terminal phase of the plasma concentration-time profile, k_e, was estimated from the terminal slope of the log-linear plot of individual plasma concentrations *versus* time. The terminal half-life ($t_{½}$) was calculated using $t_{½} = \ln 2/k_e$. The mean residence time (MRT) was calculated as the ratio of area under the first moment curve (AUMC) divided by AUC. V_{ss} was calculated as CL×MRT. The volume of distribution (V_{area}) was calculated using V_{area} = clearance/k_e.

PK Model

PK model parameters were estimated using the nonlinear least squares regression software program WinNonlin version 5.2 (Pharsight Corporation, Mountain View, California) using the Gauss Newton algorithm and uniform weighting. The PK model was selected based on goodness of fit using the Akaike's Information Criterion (AIC) and Schwarz Criterion (SC)(86) residual analysis, and overall correlation coefficient. Plasma concentration-time data for each rat were fitted using a two-compartment open body model described by $C = Ae^{-\alpha t} + Be^{-\beta t}$, where C is the total plasma concentration, t is the time in hours, A is the y axis intercept for the distribution phase, B is the y axis intercept for the linear elimination phase.

Statistical Analysis

Statistical analysis of PK parameters was performed using unpaired Student's t-test where a p value ≤ 0.05 was considered significant.

Time-Kill Studies

Previous time-kill data of MU1140 *vs*. *S. aureus* (29) were used. The time-kill studies were performed as described earlier. Briefly, bacterial inocula were prepared from test organisms grown for 4–6 h in the appropriate broth media and diluted in saline to 0.5 McFarland 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 cm^2 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. aureus* strain ONI33. The 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 hours following addition of MU1140. The samples were serially diluted 10-fold in ice cold normal saline and 10 µl samples spotted onto duplicate BAPs. Following incubation at 37°C for 24 h, colonies that arose on plates with 30–300 colonies were counted.

PD Model

Observed from previous studies of the time-kill profile of MU1140 is the re-growth of *S. aureus* after the sharp decline of the inoculum starting number(29). A common approach to model such data is the use of the two subpopulation model which assumes that the initial bacterial population was composed of two subpopulations with different susceptibility to MU1140 (designated by two different EC_{50}). The rise in bacterial numbers at later time points was explained by the continued growth of the less susceptible subpopulation. The model (10) we used assumes equal growth rate constant

and MU1140 maximum kill rate for susceptible (S) and resistant (R) subpopulations. The following differential equations describe the changes of both populations over time.

$$\frac{dS}{dt} = S(\frac{K_g \times S \times (N_{\max} - N)}{N_{\max}} - K_{\max} \frac{C}{C + EC_{50S}})$$

$$\frac{dR}{dt} = R(\frac{K_g \times R \times (N_{\max} - N)}{N_{\max}} - K_{\max} \frac{C}{C + EC_{50R}})$$

$$N = S + R$$

Where S and R are the numbers of susceptible and resistant bacteria at time t, k_g is the microbial net growth rate constant, N_{max} is maximum achievable number of bacteria in the *in vitro* medium, K_{max} is the antibiotic maximum kill rate, EC_{50} is the MU1140 concentration that produces 50% of maximal killing, and C is the concentration of MU1140 at time t.

PK/PD Model and Simulation

The PK/PD model was assembled using the developed PD model and substitution of the concentration term in the PD model with the concentration function from the pharmacokinetic model(30). The scheme of the model is presented in Figure 4-3. *V1* and *V2* are the MU1140 volumes of distribution in the central and peripheral compartments, k_{10} is the elimination rate constant, k_{12} and k_{21} are the rate constants for the transfer of MU1140 from the central to the peripheral and from the peripheral to the central compartments, respectively.

The simulation function of WinNonlin Regression Analysis software was used to simulate the concentration of *S. aureus* resulting from a certain dosing regimen and thus in designing a dosing regimen for MU1140 to be used in this *S. aureus* infection model. MU1140 concentrations resulting from two dosing regimens, 5mg/kg TID and 10mg/kg

TID were simulated. Model parameters used in the simulation were as follows: The model parameters, $V1$, $V2$, k_{12}, k_{21}, k_{10}, N, kg, EC_{50s}, EC_{50R}, K_{max} were estimated using WinNonlin.

Results

The plasma concentration-time profile of MU1140 in Sprague Dawley rats after a single IV bolus dose at 25 mg/kg is presented in Figure 4-1. MU1140 was measurable in plasma for up to 6 hours post administration. MU1140 plasma concentration-time profile declined in a biexponential mode post administration.

Noncompartmental Data Analysis

MU1140 PK parameters estimated by noncompartmental analysis of the MU1140 plasma concentrations are summarized in Table 1.

To test the linearity of the estimated PK parameters, the PK study was repeated using a MU1140 dose equivalent to 12.5 mg/kg rat body weight. For both dose groups, there was no statistical significance between CL, $t_{½}$, V_{ss}, or V_{area}. On the other hand, C_{max} and $AUC_{0-\infty}$ were dose linear. Data is summarized in table 1.

Compartmental Data Analysis of MU1140 PK Data

MU1140 plasma concentration-time data were fitted using the two-compartment open model equation. The log concentration-time profiles were characterized by a short (less than 30 minutes) but clear distribution phase followed by a slower elimination phase, as seen in Figure 4-1. The mean half-life of the distribution phase ($t_{½\alpha}$) was 4.2 min, while the terminal elimination phase mean half life ($t_{½\beta}$) was 1.6 hours. Using compartmental analysis the volume of the central compartment ($V1$) was 0.37 ± 0.23 L/kg and the volume of the peripheral compartments was estimated to be 1.4 ± 0.56 L/kg. CL and CLD2 were estimated to be 1.67 ± 0.56 L/h/kg and 2.55 ± 1.29 L/h/kg.

Time-Kill Data and PD Model

The kill profile of MU1140 against *S. aureus* is characterized by a rapid and significant decline (> 3 log drop) in bacterial counts within the first 2 hours independent of the antibiotic concentration. Regrowth is observed at all MU1140 concentrations. The experimentally observed time course of the bacterial concentrations (CFU/ml) and the bacterial concentrations predicted by PD model are presented in Figure 4-4. The model accurately predicted the bacterial concentration for all tested MU1140 concentrations. Estimates of the PD model parameters are *S. aureus* growth rate of 0.25 hr^{-1}, EC_{50S} and EC_{50R} were estimated to be 0.0001 and 2 μg/ml, respectively.

PK/PD Model and Simulation

The PK/PD model and the simulation function of WinNonlin was used to predict the concentration of viable *S. aureus* cells when MU1140 is administered and its concentration is decaying as per the pharmacokinetic model developed in rats. Figure 4-5 shows the simulated *S. aureus* counts resulting from various MU1140 dosing regimens.

Discussion

Vancomycin, the first peptide antibiotic to be used in the clinic and the current drug of last resort, is loosing ground with the emergence of vancomycin resistant *S. aureus* and *E. faecalis*. The spread of community and hospital acquired infections due to drug resistant Gram positive pathogens stresses the need for new antibiotics with novel mechanisms of action. MU1140 is active against a wide range of Gram positive organisms, including MRSA, VRE, and VISA. MU1140's unique mechanism of action involves binding and translocation of lipid II away from cell division septa. Due to its unique MOA on cell wall biosynthesis, there is a chance that MU1140 will not be subject to commonly known mechanisms of antibiotic resistance. In a previous study (29) *S.*

aureus and *S. pneumoniae* were unable to develop resistance to MU1140 after continuous sub-culturing in sub-inhibitory concentrations of MU1140 for 21 days, just a merely 3 fold increase in MIC values.

The PK profile of MU1140 was investigated after administration of an intravenous dose equivalent to 12.5 and 25 mg/kg. After intravenous administration of MU1140, its disposition was consistent with a two-compartment open model with elimination from the central compartment. The C_{max} achieved after a 25mg/kg dose is approximately 20 μg/ml and the concentration drops below 1 μg/ml within four hours, with a relatively short half-life (1.7 hr).

Noncompartmental analysis revealed that the mean volume of distribution of MU1140 in rats was about 3500 ml/kg. Given that the extracellular fluid volume of a Sprague Dawley rat is around 320 ml/kg, MU1140's volume of distribution is more than 10 times the rat's total body water. This indicates partitioning of MU1140 to blood cells, tissues and other extravascular sites.

Rapid injection of MU1140 was not well tolerated. A hypersensitivity reaction, similar to stimulation of histamine release, is observed within 5 minutes post-administration of the first dose. This reaction is characterized by redness of the ears and paws, swelling, and lethargy, and usually will last for no more than 20 minutes, after which the condition subsides. Subcutaneous administration of diphenhydramine (DPA, 20mg/kg) 1 hour before dosing of MU1140 is enough to block most of these symptoms. A similar reaction, known as "red man syndrome", is observed when vancomycin is introduced as a rapid infusion (76). In a clinical setting, pre-medication with DPA or

administration of MU1140 as a slow infusion might be needed to block or avoid this reaction.

The development of a mathematical model to describe the killing behavior allows quantitative correlation of the antibiotic concentration and the bacterial concentration at any time point. Thus, a rational dosing regimen can be conceptualized. A PD model that can explain the time-kill experiment data where MU1140 was maintained at a constant concentration was developed. Although the model fit the observed data well, such a simple model has limited utility in an in vivo scenario where the antibiotic concentration is changing. Thus, the PD model was extended for the simulation of *S. aureus* counts when MU1140 concentrations are fluctuating in a way that mirrors the plasma concentration-time profile of MU1140 observed in Sprague Dawley rats. The values of the PK/PD model parameters (Emax, kg, EC50S, and EC50R) were fixed to the values estimated from the PD model derived from the time-kill data. The model may be a useful guide to identify target PK/PD indices predictive of therapy's success in humans as well as determining initial dosing regimens in the clinic.

Conclusions

After IV administration of MU1140 in male Sprague Dawley rats, MU1140 showed two-compartmental model plasma kinetics. The developed PK model is predictive of MU1140 concentrations in Sprague Dawley rats following intravenous administration. A PD model to describe the activity profile of MU1140 against *S. aureus* using time-kill data was developed. The goodness of the model was tested by curve fitting to obtain the *S. aureus* and MU1140 specific parameter estimates. The applicability and usefulness of the PK model was enhanced by incorporating it into the

developed PD model to create the PK/PD model of MU1140 activity. Using this model, alterations in dose, and dosing regimen on the outcome of therapy can be evaluated by simulation.

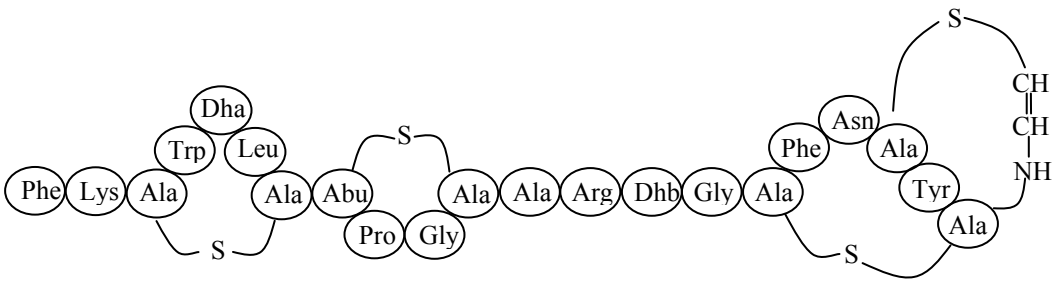


Figure 4-1. MU1140 (37)

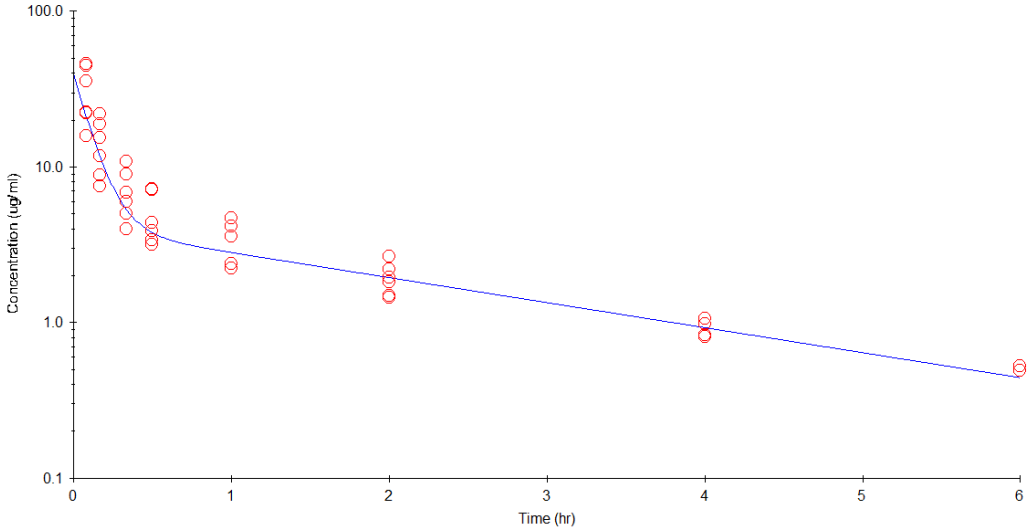


Figure 4-2. MU1140 PK profile after administration of 25 mg of MU1140 per kg rat body weight (symbols). Shown also is the simulated plasma concentration-time profile generated by the PK model (line).

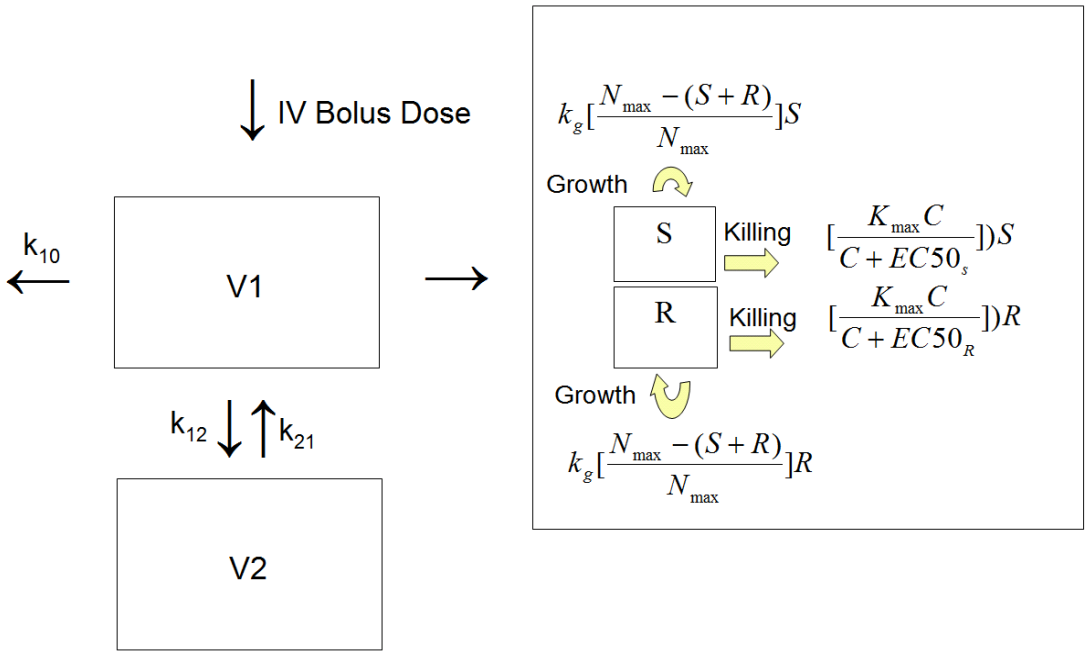


Figure 4-3. Scheme of PK/PD model for antibacterial effect of MU1140.

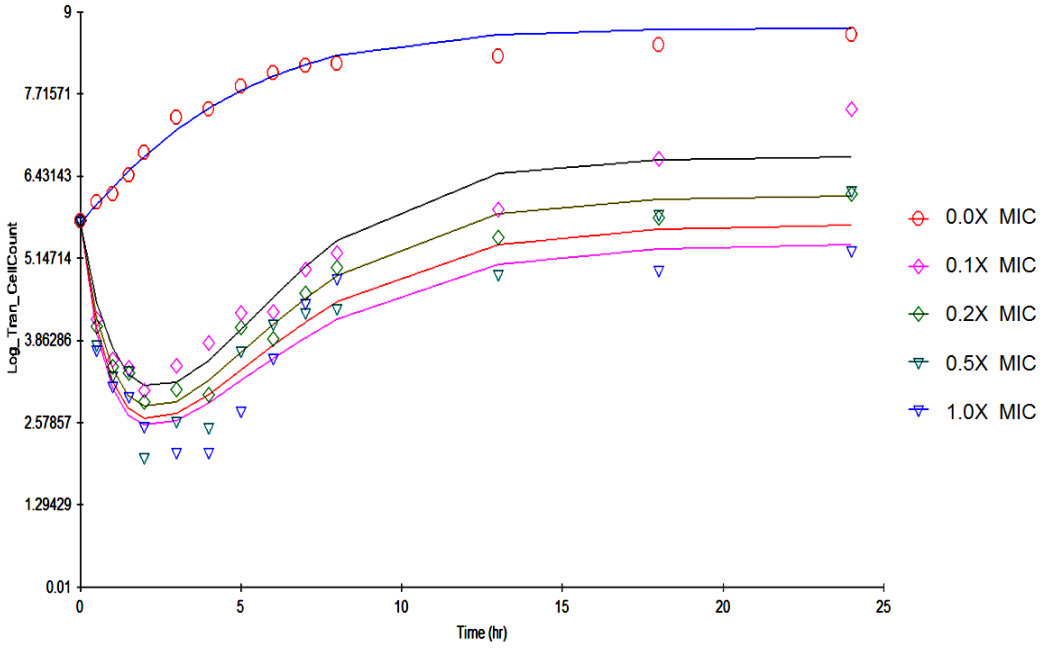


Figure 4-4. Observed vs. predicted *S. aureus* concentration (cfu/ml).

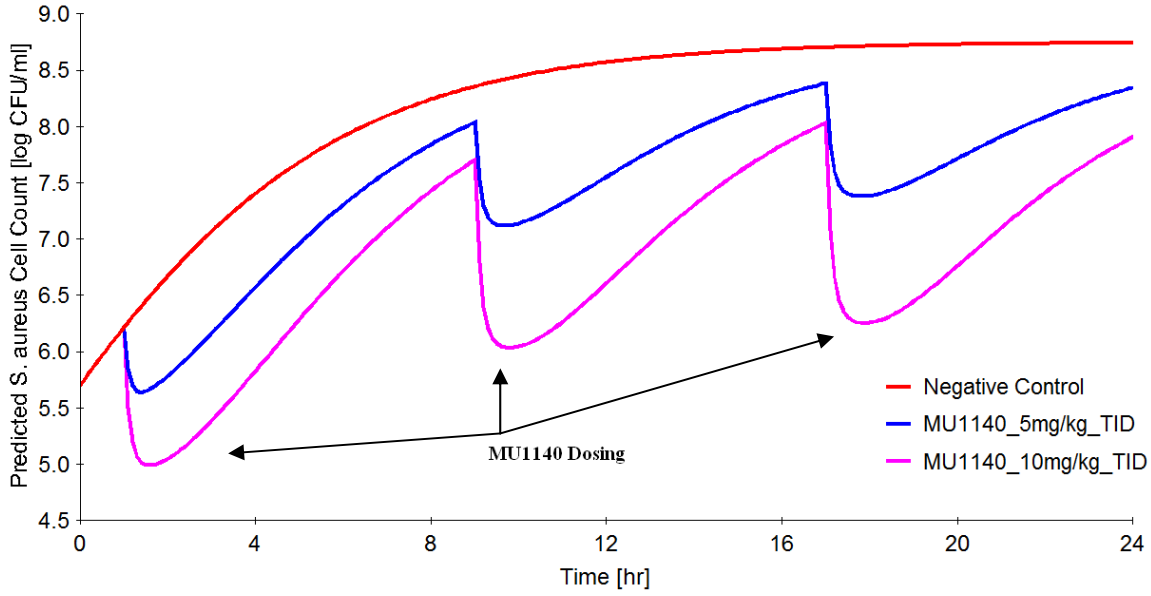


Figure 4-5. The result of the simulation of *S. aureus* viable cell count when MU1140 is administered at two dose levels (5 and 10mg/kg TID). The simulated rat PK profile was taken into consideration.

Table 4-1. Noncompartmental analysis of MU1140 (12.5, and 25mg/kg) concentration-time data. PK parameters estimated were AUC, Cl, $t_{½}$, V_c and C_{max}.

	$AUC_{0-\infty}$ (µg.hr/ml)	Clearance (ml/hr/kg)	$t_{½}$ (hr)	V_{ss}* (ml/kg)	V_{area}† (ml/kg)	C_{max} (µg/ml)
12.5mg/kg	8.7 (±4.1)	1343.1 (±212.3)	1.46 (±0.2)	2207.1 (±1103.5)	2768.6 (±1028.6)	10.7 (±4.1)
25mg/kg	18.7 (±5.5)	1441.4 (±420.7)	1.7 (±0.1)	2283.7 (±1010.5)	3478.3 (±1135.6)	31.1 (±12.6)

* V_{ss} is the estimated volume of distribution at steady state calculated as $MRT_{0-\infty} \times Cl$, based on the last observed concentration.

† V_{area} is the volume of distribution, calculated as Cl/k_e.

CHAPTER 5
PHARMACODYNAMIC ACTIVITY OF THE LANTIBIOTIC MU1140

This study evaluated the *in vitro* pharmacodynamics of the lantibiotic MU1140 as well as the ability of selected agents to develop resistance to this novel antibiotic. Susceptibility to MU1140 of over 30 Gram positive and 32 Gram negative bacteria representing 28 species was assessed. MU1140 demonstrated high activity against all tested Gram positive organisms including *Streptococcus pyogenes, Streptococcus pneumoniae, Listeria monocytogenes, and Staphylococcus aureus* and moderate activity was observed against *Enterococcus faecalis* as well as Bacillus species. MU1140 showed antimicrobial activity against oxacillin and vancomycin resistant *S. aureus*, as well as vancomycin resistant *E. faecalis* and *E. faecium*. No antibacterial activity was observed at the concentrations tested against Gram negative bacteria and yeast. Time–kill studies were used to assess the kill profile of MU1140 against clinical isolates of multidrug resistant *S. aureus*, vancomycin resistant *E. faecalis*, and an ATCC *S. pneumoniae* strain. According to CLSI susceptibility breakpoints, MU1140 was bactericidal against *S. pneumoniae* as well as multidrug resistant *S. aureus*, and bacteriostatic against vancomycin resistant *E. faecalis*. In vitro resistance development of *S. aureus* and *S. pneumoniae* to MU1140 was tested by sequential subculturing of the microorganisms in media containing subinhibitory concentrations of MU1140. After 21 subculturing events, the MIC of *S. aureus* and *S. pneumoniae* increased by only three fold. Subsequent subculturing of the strains with elevated MIC values in antibiotic free media for 7 days did not result in a reduction of their MU1140 MIC values. Collectively these findings illustrate the potential of MU1140 to serve as a therapeutic agent for the management of infections caused by Gram positive bacteria.

Introduction

The ability of microorganisms to develop resistance to antimicrobial agents has recently limited our ability to effectively control certain microbial infections and hindered effective antibiotic therapy. Infections due to antibiotic resistant pathogens have become a current, major health crisis in the world (1, 64). The situation has dramatically deteriorated during the last two decades since excessive and improper use of existing antibiotics has created multi-drug resistant bacteria, also known as the "super-bugs". In addition, discovery and development of new antibiotics has dwindled during this period. As a consequence, infections caused by these multi-drug resistant pathogens have emerged as a major cause of morbidity and mortality in the United States and worldwide (1, 81). This problem is particularly apparent in the cases of methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE). About 500,000 infections caused by MRSA are acquired in hospitals every year, costing about 8 billion dollars to treat (87). Treatment of these infections will be a major challenge in the near future and will require the discovery and development of novel antimicrobial agents (87).

A novel class of antibiotics that has long attracted much attention is the antimicrobial peptides. Antimicrobial peptides have emerged as potential therapeutic agents for the treatment of various types of bacterial infections due 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 (6, 9, 34, 90). Some of these peptides were also shown to inhibit enveloped viruses replication (57). So far, all discovered antimicrobial peptides share certain similar structural characteristics required for their bioactivity, which include an overall positive charge inferred by the presence of

multiple arginine and lysine amino acid residues as well as ~50% of the peptide's overall primary structure is composed of hydrophobic residues (90). 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 (69).

A promising class of the antimicrobial peptides are the lantibiotics. Lantibiotics (lanthionine-containing antibiotic) are peptides with antimicrobial properties that are secreted by certain Gram positive bacteria (13). Although to date, lantibiotics have not been utilized as pharmaceutical agents, several have been used in commercial applications. Nisin, for example, is a lantibiotic produced by the bacterium *Lactococcus lactis,* which has been used extensively as food preservative since the 1920's.

Lantibiotics are ribosomally synthesized and then undergo extensive post-translational modification. Lantibiotics are characterized by unusual amino acids such as lanthionine (Lan, ala-S-ala), methyllanthionine (MeLan, abu-S-ala), didehydroalanine (Dha) and didehydrobuterine (Dhb). MU1140 (Mutacin 1140, Figure 5-1) is a 22 amino acid lantibiotic that is produced by *Streptococcus mutans* (37). It has been extensively characterized in regard to its physical and chemical properties (37, 78) and its role in promoting colonization of the oral cavity by the producer strain. Its unique mechanism of action involves inhibition of the peptidoglycan synthesis by binding to and abducting lipid II from its site of action at points of peptidoglycan synthesis (35).

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 the potential of their resistance development to MU1140. Measure of the MIC was used to assess the

organisms' susceptibility to MU1140 and time-kill studies provided a dynamic picture of MU1140 antimicrobial action.

Materials and Methods

Bacteria and Media

Bacterial strains, shown in Tables 1, 2 and 3, used in the spectrum of activity studies were clinical isolates as well as ATCC strains. Kill curves were performed using a multidrug resistant strain of *Staphylococcus aureus* (ONI33) and a multidrug resistant 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* (ATCC49619). Strains ONI33 and ATCC49619 were also used in the development of resistance study. *S. aureus* strain ONI33 was shown to be resistant to amoxicillin, ampicillin, cefazolin, cefepime, cefotaxime, ceftriaxone, cefuroxime, cephalothin, ciprofloxacin, clindamycin, erythromycin, imipenem, levofloxacin, meropenem, oxacillin, penicillin, sparfloxacin, ticarcillin, azithromycin, amikacin and chloramphenicol. *E. 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 (BAP) consisting of casein peptone (1.5%; Remel, Lenexa, KS), soy peptone (0.5%; Remel), sodium chloride (0.5%; Remel), sheep's blood (5%, Lampire, PA) and agar (1.5%, Fisher, NJ). *S. aureus* strain ONI33 and *E. faecalis* strain ONI47 were grown in cation-adjusted Muller-Hinton broth (CAMH; Becton Dickinson Biosciences, Franklin Lakes, NJ) at 37°C in a CO_2 (5%) incubator. *S. pneumoniae* strain ATCC49619 was

grown in Todd-Hewitt broth (THB; Becton Dickinson Biosciences) under the same conditions.

Antimicrobial Agents

MU1140 was manufactured by Oragenics, Inc. Purity was estimated to be greater than 90% as determined by analytical reverse phase HPLC (RP-HPLC).

Susceptibility Studies

The minimum inhibitory concentrations (MIC) of MU1140 against target microorganisms were determined by Focus Bio-Inova (Herndon, VA). Aerobes MU1140 MIC values were determined by broth microdilution method according to CLSI-defined methodology (M7-A6), while MU1140 MIC values for anaerobes were determined by the agar dilution according to CLSI-defined methodology (M11-A5).

Time–Kill Studies

The MICs for *S. aureus* strain ONI33, *E. faecalis* strain ONI47 and *S. pneumoniae* strain ATCC49619 used in the time-kill and development of resistance studies were determined using the microbroth dilution 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 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 cm^2 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 ATCC49619 and *S. aureus* strain ONI33, and 0.25, 0.5, 1, 2, 4, 8, and 16 times the MIC for *E. fecalis* 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 hours following addition of MU1140. The samples were serially diluted 10-fold in ice cold normal saline and 10 μl samples spotted onto duplicate BAPs. Following incubation at 37°C for 24 h, colonies that arose on plates with 30–300 colonies were counted.

Development of Resistance

S. 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. Cells were then diluted 1:100 in appropriate broth media to give approximately 10^6 cfu/ml, and 100 μl samples were added to microtiter wells (Corning Inc, Corning, NY) containing 100 μl of doubling concentrations of MU1140 in broth to achieve a final bacterial concentration of 5×10^5 cfu/ml. The microtiter plates were incubated overnight at 37°C in an atmosphere of 5% CO_2. Wells containing the highest concentration of MU1140 that showed turbidity (equivalent to 0.5 MIC) were diluted to 0.5 McFarland 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 7^{th}, 14^{th}, and 21^{st} 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 6 times, after which MICs for MU1140 were determined using the broth microdilution method.

Results

Susceptibility Studies

The results of the tiers 1 and 2 susceptibility studies are summarized 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 – 32 μg/ml. It was most potent against *Streptococcus pyogenes* (MIC is 0.5 μg/ml) and least potent against *E. fecalis* and *E. faecium* (MICs range 16 – 32 μg/ml). In the tier 2 studies (Table 2), MU1140 showed greater activity (MIC <8 μg/ml) against *S. pyogenes, L. monocytogenes,* and *C. difficile* than against *S. aureus*, *E. fecalis*, and *Bacillus sp*. (MIC >8 μg/ml). Results of the tier 3 studies (Table 3) revealed that MU1140 was as active as vancomycin against vancomycin intermediate *S. aureus*, but showed superiority to vancomycin against all tested vancomycin resistant *S. aureus*, and vancomycin resistant *E. faecalis* and *E. faecium*. Vancomycin had lower MIC values when compared to MU1140 when tested against all vancomycin sensitive strains.

Time-Kill Studies

One isolate each of *S. pneumoniae* (Figure 5-2), MDR *S. aureus* (Figure 5-3) and VRE (Figure 5-4) were selected as test organisms for the time-kill analysis. Very similar kill profiles were observed for *S. pneumoniae* and *S. aureus*, characterized by a rapid and significant decline (> 3 log drop) in bacterial counts within the first 2 hours independent of the antibiotic concentration. Regrowth is observed at lower MU1140 concentrations (0.5X, 1X MIC for *S. pneumoniae*, and 0.5X, 1X, 2X MIC for *S. aureus*) but not in concentration > 8X MIC for all strains. For *S. pneumoniae*, time to 99.9% killing after exposure to MU1140 at 1 and 2 times MIC (1X and 2X MIC) was 5 hrs while at 4 and 8 times MIC (4X and 8X MIC) was 2.5 hrs. For MDR *S. aureus*, time to 99.9% killing after exposure to MU1140 at 0.5 times MIC (0.5X MIC) was 1.5 hrs and at 4, 8, and 16 times MIC (4X, 8X, 16X MIC) was 0.5 hrs. CLSI defines a bactericidal agent as one that 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 0–3 $\log_{10}$ cfu/ml.

According to that definition, time-kill studies reveal that MU1140 is bactericidal against *S. pneumoniae* at concentrations at or above 1X MIC and bactericidal against *S. aureus* at concentrations at 0.5X MIC or above. The time-kill studies also reveal that MU1140 is bacteriostatic against vancomycin resistant *E. fecalis* (Figure 5-4) at all concentrations tested and maintained bacterial counts at approximately the initial inoculum size.

Resistance Development Study

MIC values resulting from daily subculturing of *S. aureus* strain ONI33 and *S. pneumoniae* strain ATCC 49619 are summarized in Figure 5-6. Sequential subculturing of the these strains resulted in emergence of variant stains with elevated MU1140 MIC values. The MICs for the parent *S. aureus* was 3.2 μg/ml and doubled after the second and the twelfth subculturing to stabilize at 12.8 μg/ml. The MIC of MU1140 against the *S. pneumoniae* parent started at 0.4 μg/ml and doubled after the second, third, and fourth subculturing event, stabilizing at 3.2ug/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.

Discussion

The rise in bacterial resistance to currently used antibiotics is an alarming reality that has attracted considerable worldwide attention (84). The emergence of antimicrobial resistance is not a new phenomenon. It is an inevitable result of the large number of bacterial species, their rapid replication, and the frequent and misuse of antibiotics. A mutation or acquisition of a gene that helps a microbe survive in the presence of an antimicrobial agent will quickly become predominant throughout the microbial population and may spread from person to person. Clearly, there is a need for more sensible use of existing antibiotics, but more importantly there is a pressing need for the

development of new antibiotics. The class of antibiotics known as lantibiotics has been known for decades, and throughout this period many investigators, reviewed by cotter et al. (15), have predicted their potential for use as therapeutic agents. The goal of this study was to evaluate 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 breakpoint definitions (58), while none of the 28 Gram negative species or the yeast species tested showed sensitivity. These findings are in accord with previously reported studies (36, 37). The lack of Gram negative bacteria and yeast sensitivity to MU1140 is likely to be a function of it's mode of action. MU1140 exerts its antimicrobial effect by a novel mechanism (88), 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 ones 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. feacium* being

less susceptible. At present, there is no definitive explanation for these observed differences.

Although MIC determination is still the gold standard for characterizing 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 (55), which were performed using strains of medically important Gram positive species, *S. aureus, S. pneumoniae,* and *E. faecalis*. The results of time-kill investigations showed that MU1140 exhibit rapid initial killing against MDRSA and *S. pneumoniae*, whereas a bacteriostatic activity was observed against a vancomycin resistant strain of *E. faecalis*. Vancomycin also exhibits this species-dependent difference in activity (2, 25). 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, MDRSA and *S. pneumoniae* mutants with modest 3-fold elevated MU1140 MICs were selected. This phenotype was stable, indicating the selection of genetic variants. Resistance development to lantibiotic has been extensively studied using nisin, reviewed by Chatterjee (13), 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, there was 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 due to the rise of drug resistant pathogens. Low level increase in 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 of *Streptococcus mutans,* JH1140, has an MIC comparable to other streptococci, indicating that it has not been able to develop effective immunity against its own bacteriocin. Additional work is in progress to determine the usefulness of MU1140 as a clinically useful therapeutic agent for the treatment of infectious diseases.

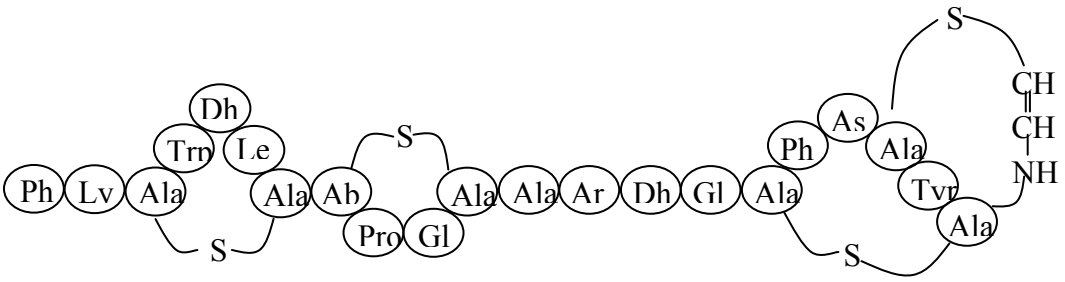


Figure 5-1. MU1140

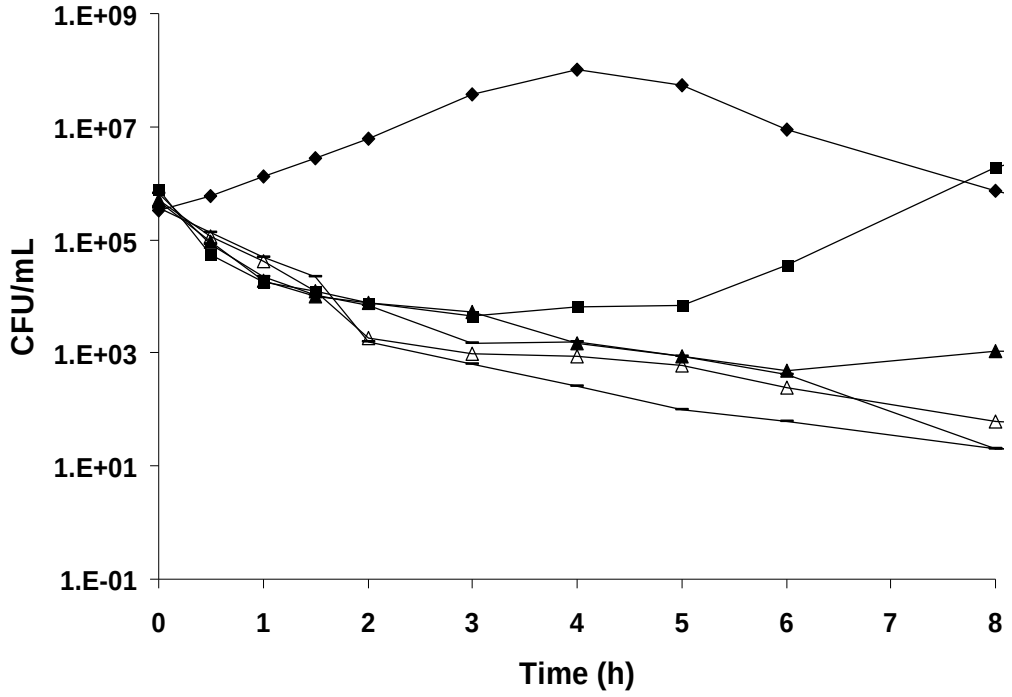


Figure 5-2. Bactericidal activity of MU1140 against *S. pneumonia* strain ATCC 49619. Symbols: ♦ Control, ■ 0.5×MIC, ▲ 1×MIC, │ 2×MIC, Δ 4×MIC, ▬ 8×MIC.

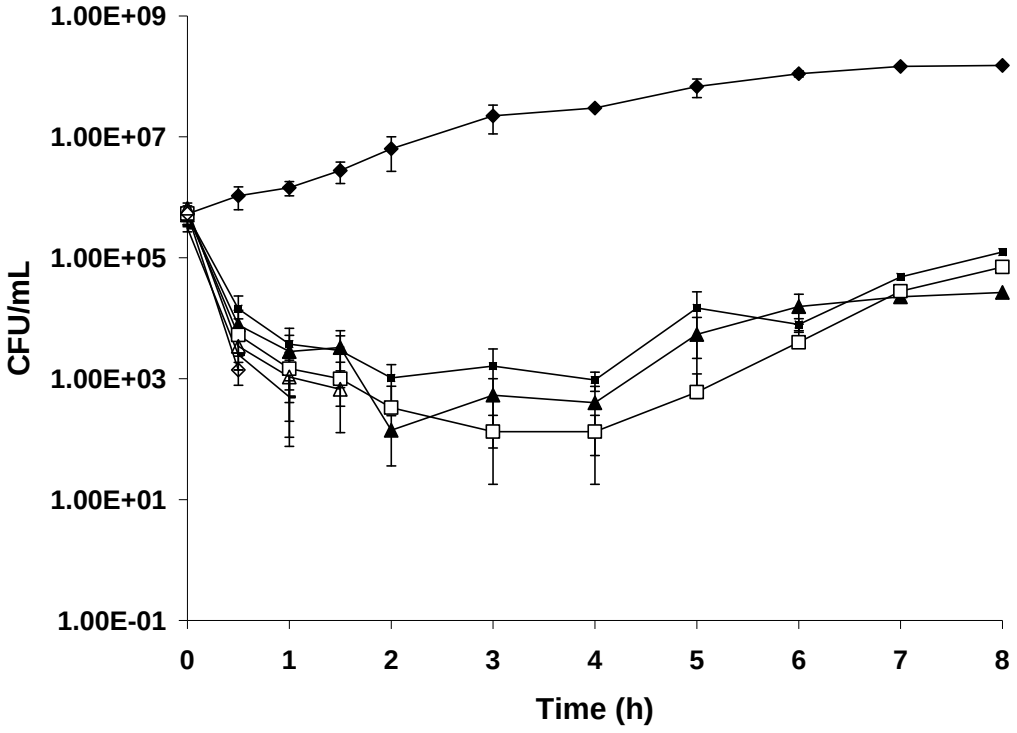


Figure 5-3. Bactericidal activity of MU1140 against multidrug resistant *S. aureus*. Symbols: ♦ Control, ■ 0.5×MIC, ▲ 1×MIC, □ 2×MIC, Δ 4×MIC, ▬ 8×MIC, ◊ 16×MIC

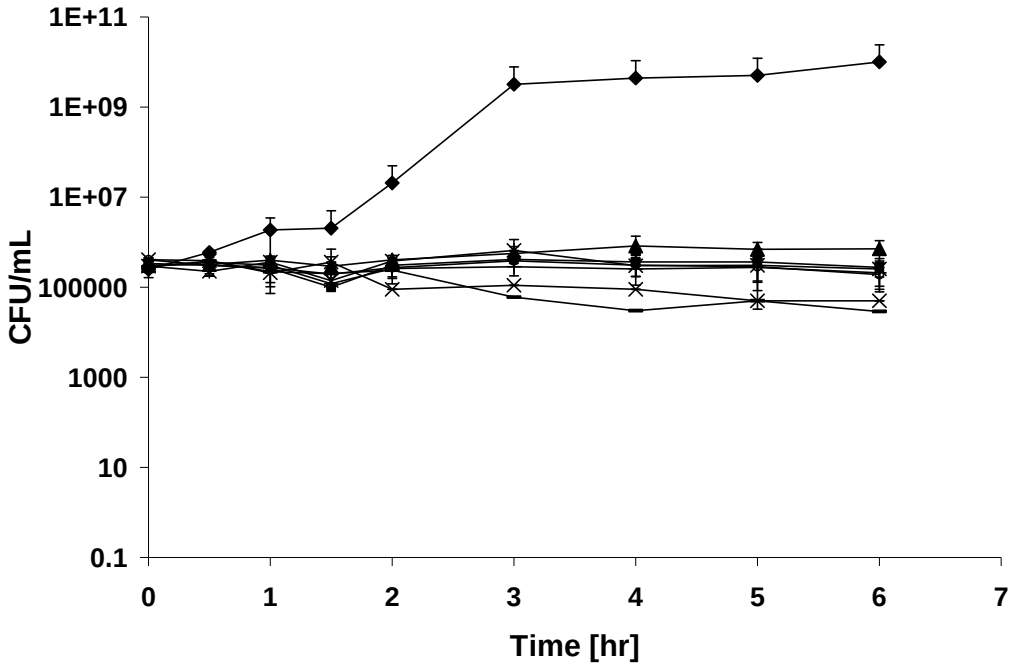


Figure 5-4. Bacteriostatic activity of MU1140 against vancomycin resistant *E. faecalis*. Symbols: ♦ Control, x 0.25×MIC, ■ 0.5×MIC, ▲ 1×MIC, □ 2×MIC, Δ 4×MIC, — 8×MIC, ◊ 16×MIC

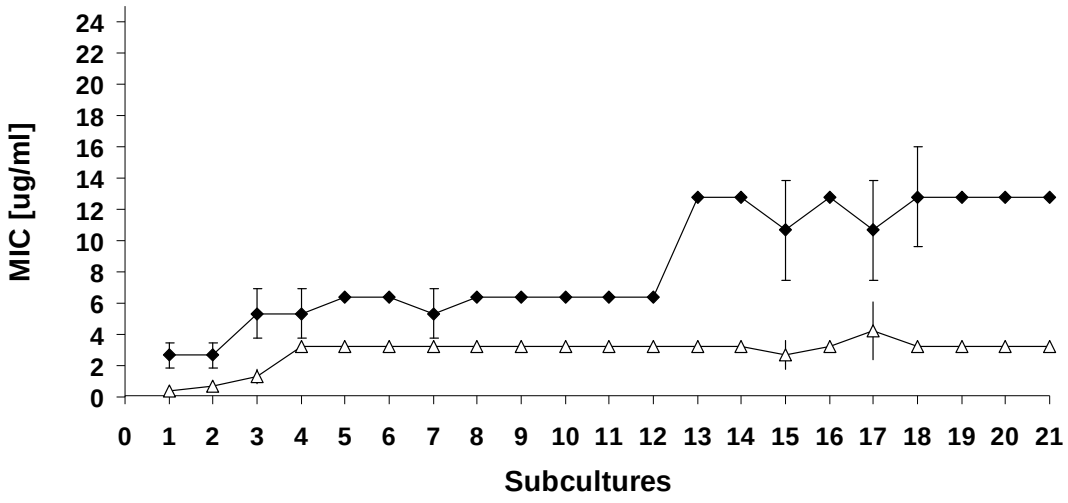


Figure 5-5. MU1140 MIC values after 21 subculturing events for multidrug resistant *S. aureus* (♦) and *S. pneumoniae* (Δ). Decrease in susceptibility after repeated subculturing in subinhibitory MU1140 concentrations is evident by the increase in organisms' MU1140 MIC values.

Table 5-1. Tier 1 susceptibility Study. MU1140 MIC for various Gram positive and negative microorganisms, and yeast.

Microorganism (number of isolates)	MU1140 MIC (μg/ml)
Gram Positive Organisms	
Enterococcus faecalis (3)	16-32
ATCC 29212 *Enterococcus faecalis* (1)	32
Enterococcus faecium (4)	8-32
Staphylococcus aureus (4)	16
ATCC 29213 *Staphylococcus aureus* (1)	16
Staphylococcus epidermidis (4)	16
Staphylococcus saprophyticus (2)	4-16
Streptococcus agalactiae (2)	4
Streptococcus intermedius (1)	2
Streptococcus mitis (1)	4
Streptococcus pneumoniae (3)	1
ATCC 49619 *Streptococcus pneumoniae* (1)	4
Streptococcus pyogenes (2)	0.5
Clostridium difficile (2)	1
Gram Negative Organisms	
Acinetobacter baumannii (2)	>32
Acinetobacter calcoaceticus (2)	32
Citrobacter freundii (2)	>32
Citrobacter koseri (diversus) (2)	>32
Enterobacter cloacae (2)	>32
ATCC 25922 *Escherichia coli* (1)	>32
Haemophilus influenzae (1)	>32
ATCC 49247 *Haemophilus influenzae*	>32
Klebsiella oxytoca (2)	>32
Klebsiella pneumoniae (2)	>32
Morganella morganii (2)	>32
Proteus mirabilis (2)	>32
Proteus vulgaris (2)	>32
Providencia stuartii (2)	>32
Pseudomonas aeruginosa (1)	>32
ATCC 27853 *Pseudomonas aeruginosa*	>32
Serratia marcescens (2)	>32
Stenotrophomonas (Xanthomonas) maltophilia (2)	>32
ATCC 25285 *Bacteroides fragilis*	>32
Yeast	
Candida albicans (1)	>32
ATCC 90028 *Candida albicans* (1)	>32

Table 5-2. Tier 2 susceptibility Study. MU1140 MIC for various Gram positive and anaerobic microorganism

Microoganism (number of isolates)	Antibiotic MIC (μg/ml)	
	MU1140	Vancomycin
Enterococcus faecalis		
Vancomycin S (9)	16-32	1-2
Vancomycin R (9)	16	>64
Streptococcus pyogenes		
Erythromycin (S)	0.5-2	0.5
Erythromycin (R)	0.5-1	0.5
Staphylococcus aureus		
Vancomycin S MRSA (9)	8-32	1
Inpatient Vancomycin S MRSA (10)	16-32	1
Community Acquired Vancomycin S MRSA (4)	16	1
Streptococcus pneumoniae		
Penicillin S (9)	0.5-8	0.25-0.5
Penicillin R(9)	0.25-8	0.25-0.5
Listeria monocytogenes		
(9)	4	1
(9)	16-32	0.5-2
Clostridium difficile		
(9)	0.5-2	NA
Bacillus species		
(9)	16-32	0.5-2

TABLE 5-3. Tier 3 susceptibility study. MICs of MU1140 in comparison to vancomycin against selected clinical isolates

Microorganism (number of isolates)		Antibiotic MIC (μg/ml)	
		MU1140	Vancomycin
Staphylococcus aureus			
	OXA-S (22)	2-8	0.5-1
	OXA-R(33)	2-8	0.5- >128
	VAN S (51)	2-8	0.5-2
	VAN I (1)	4-4	4-4
	VAN R (3)	4-8	>128
Enterococcus faecalis			
	VAN S (17)	4-8	0.5-4
	VAN R (14)	4-8	32- >128
Enterococcus faecium			
	VAN S (12)	2-8	0.5-1
	VAN R (13)	1-8	64- >128

CHAPTER 6
IN VITRO SERUM PROTEINS BINDING AND ITS EFFECT ON THE PHARMACODYNAMICS OF THE LANTIBIOTIC MU1140

The lantibiotic MU1140's degree of binding to serum proteins and the effect of serum components on the bactericidal activity of this novel antibiotic were investigated. The percentage of MU1140 bound to human serum proteins was determined by ultrafiltration to be 92.7% ± 2% when tested in the range of 6.25-200 μg/ml. The presence of inactivated serum increased the average MU1140 MICs for *Streptococcus pneumoniae (S. pneumoniae)*, but decreased the average MICs for *Staphylococcus aureus (S. aureus)*. Time-kill studies of MU1140 against *S. pneumoniae* and *S. aureus* in serum-containing medium showed that in serum containing media higher drug concentrations were needed to achieve the bactericidal effect against *S. pneumoniae* than were needed in broth, while lower amounts of the antibiotic were needed to achieve the bactericidal effect against *S. aureus* than were needed in broth. Pooled serum exerts a protective effect for *S. pneumoniae* against MU1140, but enhanced MU1140's antibacterial activity against *S. aureus* suggesting a possible synergistic effect between this lantibiotic and serum components.

Introduction

Bacterial resistance to currently available antibiotics is a public health crisis that deserves worldwide attention (1). The clinical selection of bacterial isolates resistant to currently used antibiotics is an evolutionary force that derives the emergence of the superbugs (64). Currently, 50% of hospital acquired infections are due to multidrug resistant bacteria which annually result in over 100,000 deaths and 100 billion dollars in associated healthcare cost (12, 41). Of special importance are the methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE),

which are very difficult to treat and contribute heavily to hospital associated deaths. Thus, the need for new antibiotics is great, especially those with novel mechanisms of action as this will ensure the lack of cross resistance to the newly developed antibiotics (16).

MU1140 is a 22 amino acid bacteriocin with a novel mechanism of action. MU1140 is produced by *Streptococcus mutans* strain JH1140 (37) and it belongs to the lantibiotics which is a group of ribosomally synthesized and posttranslationally modified bacteriocins. Lantibiotics contain unusual amino acids such as lanthionine, as well as methyllanthionine and didehydroalanine. Lanthionine amino acids are composed of two alanine residues cross-linked via a thioether linkage that connects their *β*-carbons (*S*-(alaninyl-3-yl)-cysteine) (Figure 6-1). Lantibiotics are produced by a large number of Gram-positive bacteria and have their lanthionines imbedded within cyclic peptides (13). MU1140 has been characterized in regard to its physical and chemical properties (37, 78) and its role in promoting the producer strain ability to colonize the oral cavity. Its novel mechanism of action involves inhibition of the peptidoglycan cell wall synthesis by binding to and abducting lipid II from its site of action at sites of peptidoglycan synthesis (35).

Inappropriate dosing has been implicated as one of the major factors contributing to the continuous emergence and spread of antibiotic resistant bacterial strains (73). In order to optimize dosing of a new antibiotic, all factors that determine the pharmacologically active fraction has to be taken into account, one being the degree of antibiotic binding to serum proteins. Once drug molecules enter the systemic circulation, they exist in a state of dynamic equilibrium between two forms, bound to blood

components and unbound or free. A common attachment site for drugs in the blood is serum proteins, usually albumin, forming a reversible complex by hydrogen bonding (45). Binding to serum proteins is an important property of a drug since the degree of binding determines the pharmacologically active free fraction (17, 80). Only the unbound drug is able to exert the pharmacological effect because this is the fraction of drug molecules free to diffuse and reach the biophase (80). Overall, plasma protein binding influences the disposition profile as only free drug is available for elimination and distribution into peripheral tissues. The effect of protein binding on antibiotic action has been well documented and reviewed (18, 19). Since most infections take place in various body tissues, for an antibiotic to be efficacious it has to penetrate the tissue and there it will exert its antimicrobial effect. In that sense, an antibiotic's degree of protein binding is a factor of great importance since it determines the fraction of the dose that is free to illicit its antimicrobial effect and consequently determines the outcome of therapy (85). Generally, reduction in activity is found to correlate directly with decreasing free fraction, i.e. increased percentage binding (52) and the *in vivo* activity inversely correlated with the extent of binding, thus the highly bound molecules have a much higher ED_{50} values and a longer *in vivo* half life than the compounds with lower binding coefficients. The flip side of the coin is also true, only the free drug molecules are available for clearance, either by kidney filtration, uptake and metabolism by liver cell, or any of the other clearance mechanism. Since binding to blood components can affect the drug's distribution, tissue penetration, metabolism, and elimination from the body (17, 23, 68) it can have a profound effect on the drug's pharmacodynamic and pharmacokinetic behavior (5).

The aim of this study was to investigate the degree of MU1140's binding to serum proteins and the effect of protein binding on MU1140's pharmacodynamic activity against multidrug resistant *S. aureus* clinical isolate and *S. pneumoniae* (ATCC 49619) as measured by MIC determination and time-kill studies. Although MIC can serve as an indicator of a drug's *in vitro* potency, it does not provide any data on the drug's killing kinetic profile. Time-kill curves on the other hand provide a dynamic view of the drug's antimicrobial activity that is of great clinical relevance (55). The bactericidal activity of MU1140 has been investigated (4), but the degree of protein binding and bactericidal activity in serum has not been reported for any of the lantibiotics.

Materials and Methods

Determination of MU1140's Degree of Binding to Human Serum Proteins

Human serum was spiked with MU1140 to yield final concentrations of 6.25, 12.5, 25, 50, 100, and 200µg/ml. Following incubation at 37^{o}C for 1 h, an aliquot was transferred to an ultrafiltration device with a molecular weight cut-off of 10'000 Dalton (Amicon Co., Danvers, MA, USA), which was centrifuged at 15000 ×g for 1 h at 37^{o}C. Samples' ultrafiltrate were analyzed by LC-MS. The concentrations of MU1140 in the filtrate were determined using standard samples prepared by spiking known amounts of MU1140 in plasma followed by ultrafiltration in the same fashion. The unbound fraction was estimated from the ratio of drug concentration in the filtrate to that in the original plasma samples. Parallel studies using protein-free plasma instead of plasma indicated that MU1140 was bound minimally to the ultrafiltration device.

Broth Preparation

Cation adjusted Mueller-Hinton broth (MHB; Becton Dickinson, Franklin Lakes, NJ, USA) was used to grow S. aureus, Todd Hewitt broth (THB; Difco, Detroit, USA)

was used for S. pneumonia. Both were prepared at 4 times (4X) the manufacturer's suggested concentration and autoclaved prior to use at 121oC (15 min per 1L). Sprague Dawley rat serum was purchased from Innovative Research (Southfield, MI) and human serum was purchased from Rockland Immunochemicals (Gilbertsville, PA). Serum containing media was prepared as follows; 0% serum medium contained one part of 4× broth and 3 parts of autoclaved 0.9% sodium chloride, 25% serum medium contain one part of 4× broth, one part of heat inactivated (55°C × 30 min) serum, and 2 parts of autoclaved 0.9% sodium chloride, 50% serum medium contain one part 4× broth, 2 parts of heat inactivated serum, and one part autoclaved 0.9% sodium chloride, and the 75% serum medium contain one part 4× broth and three parts of heat inactivated serum.

Bacterial Cultivation

The bacterial inocula were prepared from colonies grown overnight on blood agar plates (BAP; 5% sheep blood agar plates, Remel Microbiology Products, Lenexa, KS, USA). Cells were scraped from the plate using an inoculation loop and suspended in sterile saline solution to 0.5 McFarland units, which is equivalent to a concentration of $1x10^8$ cfu/mL.

Time-Kill Studies

Two types of time-kill studies were conducted:

1. Studies in which the amount of serum in the growth medium was fixed at 50% and varied the concentration of MU1140 (0.1-16 times MIC).
2. In the other study fixed the amount of MU1140 was 0.5 × MIC, and varied the amount of serum in the growth media (0, 25, 50, and 75%).

For all time-kill studies an *in vitro* kinetic model was used. This system consisted of eight 50 ml vented cap tissue culture flasks with canted necks (nunc™, Nunc A/S, Roskilde, Denmark), each containing 20 ml of the appropriate broth media.

For the first study, growth media was prepared as follows: 0% serum flasks contained one part of 4× broth and 3 parts of autoclaved 0.9% sodium chloride; 50% serum flasks contain one part 4× broth, 2 parts of heat inactivated serum, and one part autoclaved 0.9% sodium chloride. Untreated (not inactivates) human serum was used for this study.

For the second study the growth media was prepared as follows: 0% serum flask contained one part of 4X broth and 3 parts of autoclaved 0.9% sodium chloride; 25% serum flask contained one part of 4X broth, one part of heat inactivated (55°C for 30 min) serum, and 2 parts of autoclaved 0.9 sodium chloride; 50% serum flask contained one part 4X broth, 2 parts of heat inactivated serum, and one part autoclaved 0.9% sodium chloride; 75% serum flask contained one part 4X broth and three parts of heat inactivated serum.

A 100 µl aliquot of the 0.5 McFarland inoculum was added to each flask to produce a final inoculum of approximately $5x10^5$ CFU/mL. The bacteria were incubated standing for 2 hours at 37^oC in an atmosphere of 5% CO_2 to allow them to reach the exponential growth phase before adding different MU1140 concentrations. The selection of MU1140 concentrations tested for each bacterial strain was based on their previously determined MIC values. At least six different concentrations were investigated besides the MIC, which included subinhibitory (0.06, 0.12, 0.25 and 0.5 times MIC) and suprainhibitory concentrations (2, 4, 8 and 16 times MIC). A control with bacteria and no drug was run simultaneously. Fifty microliter samples were removed, diluted in saline, and plated at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, and 24 hours post-antibiotic addition for quantification.

Bacterial Quantification

Bacterial counts were determined by plating 50 µL of serial 10-fold dilutions in 0.9% saline on BAPs, where plates were divided into four quadrants. Using a pipette, five 10 µL droplets of the chosen dilution were spotted equidistantly onto one of the quadrants. Replicate spots were plated onto the adjacent quadrant. The plates were then incubated at 37^{o}C in 5% CO_2 for 18-24 hours before reading. Positive controls with bacteria but no drug were run simultaneously. Following incubation, colonies that arose were counted. Data were used from quadrants that contained 15-150 colonies. The experiments were performed independently in triplicate. The means and standard deviations were calculated.

Results

Determination of MU1140 Unbound Fraction in Human Serum

MU1140 was spiked in human serum to achieve concentrations of 6.25-200 µg/ml. The samples were subjected to ultrafiltration using Ultrafree®-MC Centrifugal Devices (50 KDa cut off). The average MU1140 percentage binding is 92.7%.

Effect of Protein Binding on MU1140's *In vitro* Activity: MIC Studies

The effect of inactivated human serum upon the *in vitro* activity (MICs) of MU1140 against *S. pneumoniae* and *S. aureus* was studied. A microdilution method was employed using either Muller Hinton Broth alone or Muller Hinton broth supplemented with 25 or 50% (v/v) inactivated human serum for *S. aureus*. For *S. pneumoniae*, Todd Hewitt broth (THB) or THB supplemented with 25 or 50% (v/v) inactivated human serum was used. A bacterial final inoculum of 5×10^5 cfu/mL was employed. Following incubation for 18–24 h at 35–37°C in an atmosphere enriched with 4–6% carbon dioxide for *S. pneumoniae* and *S. aureus*. The MIC was defined as the lowest concentration at

which there was no visible growth. The presence of 25 and 50% (v/v) human serum lead to a two fold and four fold increase in MU1140 MIC respectively against *S. pneumoniae*. On the other hand, the presence of 25 and 50% inactivated human serum increased MU1140 activity against S. aureus causing a reduction in the MIC value from 4.8μg/ml in 0% serum to 1.6μg/ml in the 25 and 50% serum wells. Data is summarized in tables 6-1 and 6-2.

Effect of Protein Binding On MU1140's *In vitro* Activity: Time-Kill Studies

In the presence of 50% human serum, MU1140 antibacterial activity was decreased against *S. pneumonia.* Figure 2C illustrates the significant increase in bacterial viable cell counts at all MU1140 concentration when compared to those in the absence of serum (Figure 6-2A). By plotting the viable cell counts in flasks containing equivalent MU1140 concentration in the presence and absence of serum on the same plot (Figure 6-3 panels A-H), the inhibition of antibacterial activity is apparent. It seems that the presence of serum inhibits MU1140 by four fold as shown in panel F, where the bacterial cell counts for the 1 × MIC in broth and the 4 times MIC in serum (Figure 3 panel F) are superimposable.

Human serum seems to exert the opposite effect on MU1140 antibacterial activity against *S. aureus*. Unlike the picture observed with *S. pneumoniae*, *S. aureus* viable cell count decreased dramatically in the presence of serum when compared to the viable cell count in the absence of serum. Figures 6-2B and 6-2D illustrates the decrease in bacterial cell counts at all MU1140 concentration when compared to those in the flasks with broth only. Again, by plotting the bacterial viable cell counts from flasks containing equivalent MU1140 concentration in the presence and absence of 50% serum on the same plot (Figure 6-4 panels A-H) the effect exerted by serum on MU1140 activity is more evident.

To quantify human serum's degree of augmentation of MU1140's activity, the viable cell count plots were superimposed in the presence and absence of serum. Again, the profile of 1 times MIC in serum and 4 times MIC in broth seem to be superimposable indicating that the presence of serum augments MU1140 antibacterial effect against *S. aureus* by four fold which suggests a possible synergistic effect between serum components and MU1140 against *S. aureus*.

In a further attempt to understand the correlation between the human or rat serum concentration in the growth medium and MU1140 antibacterial effect on *S. aureus* a second set of time-kill studies were performed where the bacterial inoculum ($5x10^5$ CFU/ml) and MU1140 concentration (0.5 time MIC) were fixed, but varied the human or rat serum concentrations (0, 25, 50, and 75%). As shown in Figure 6-6, the most significant kill is observed when human (Figure 6-6 panel A) or rat (Figure 6-6 panel B) serum is in small amounts (25%). There was no difference in the viable cell counts in the 50% and 75% serum flasks. The growth control of *S. aureus* in the various serum containing media are comparable, suggesting that the observed kill behavior was due to MU1140 antibacterial activity. Similar behavior is observed when testing the MU1140 kill behavior against *S. aureus* using Sprague Dawley rat serum.

Discussion

Only the free (non protein bound) fraction of an antibiotic exerts antibacterial activity and is able to diffuse rapidly from plasma to the extravascular compartments where most bacterial infections are located (11, 43, 62, 72). For highly bound drugs (percentage bound exceeds 70-80%), binding to serum proteins is a crucial factor since small changes in binding will produce large variations in the percentage of free and pharmacologically active drug (18).

The results in this study show that MU1140 is 92.7% ± 2% protein bound and that the MIC values increase proportional to the concentration of serum in growth media when tested against *S. pneumoniae.* A different picture is apparent when testing the bactericidal activity against *S. aureus* where a synergy/augmentation of bactericidal activity is noticed when serum proteins are present in the culture broth, a phenomenon that has not been reported before for any lantibiotic. Overall, it was concluded that the pharmacodynamic activity of MU1140 in the presence of serum suggests clearly that it is the free fraction of MU1140 that correlates with its antibacterial effect.

Conclusions

The serum protein binding and its effect on the bactericidal activity of MU1140 against *S. pneumoniae* and a multi drug resistant *S. aureus* was investigated. Protein binding was determined in human serum by ultrafiltration to be 92.7% ± 2% when tested in the range of 6.25-200 μg/ml. The presence of inactivated human serum (25 and 50%) increased the average MU1140 MICs for *Streptococcus pneumoniae* in a concentration dependent manner, but it decreased the average MICs for *S. aureus* by a nonproportional factor of two. Time-kill studies of MU1140 against *S. pneumoniae* and *S. aureus* in serum-containing media (25, 50, and 75%) showed that much higher drug concentrations were needed to achieve the bactericidal effect against *S. pneumoniae* than were needed in broth, while lower amounts of the antibiotic were needed to achieve a bactericidal effect against *S. aureus* than were needed in broth. The high level of protein binding of MU1140 appears to influence its antibacterial activity against *S. pneumoniae,* but a synergistic effect between MU 1140 and low concentrations of serum components is observed against *S. aureus*.

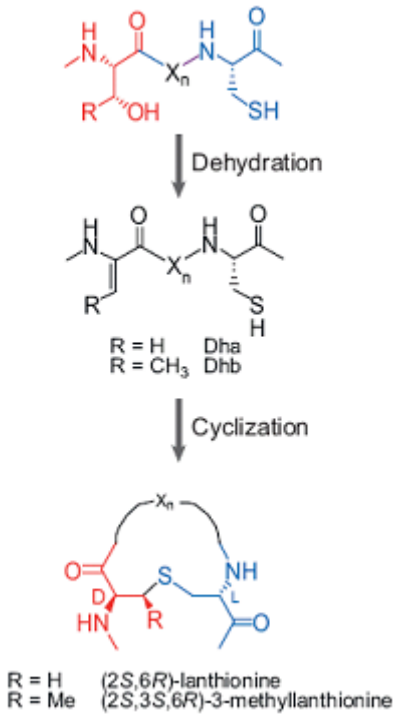
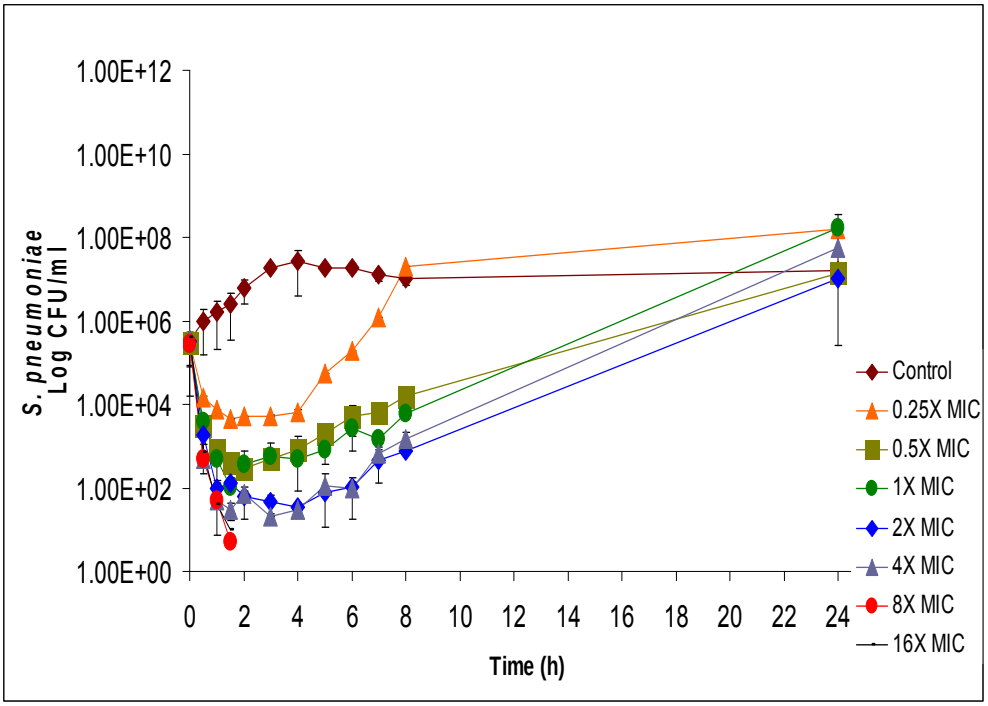
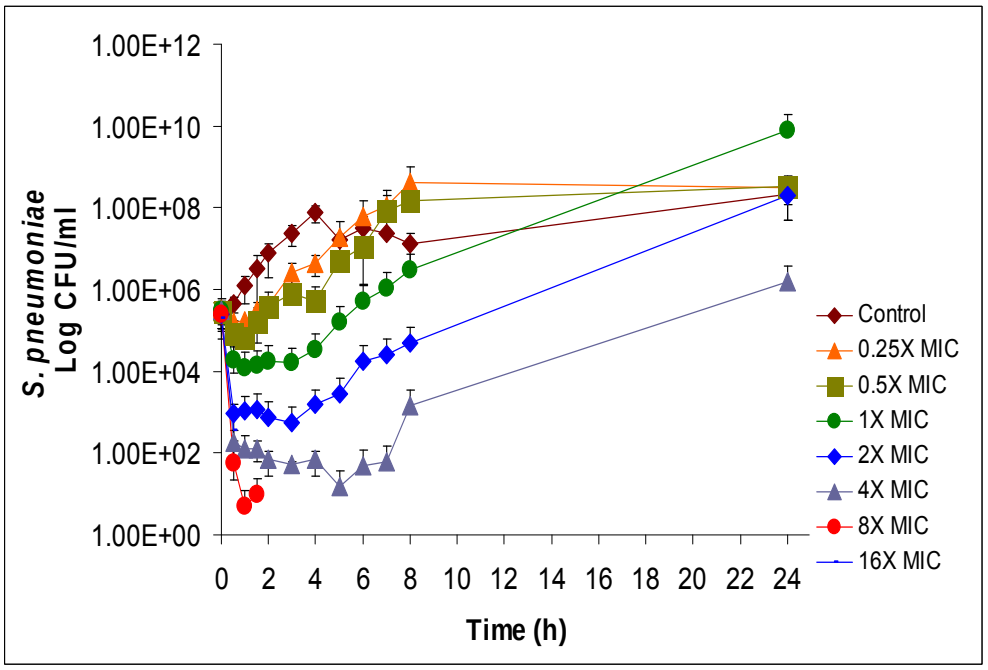


Figure 6-1. Lanthionine (Lan) and Methyllanthionine (MeLan) structure.

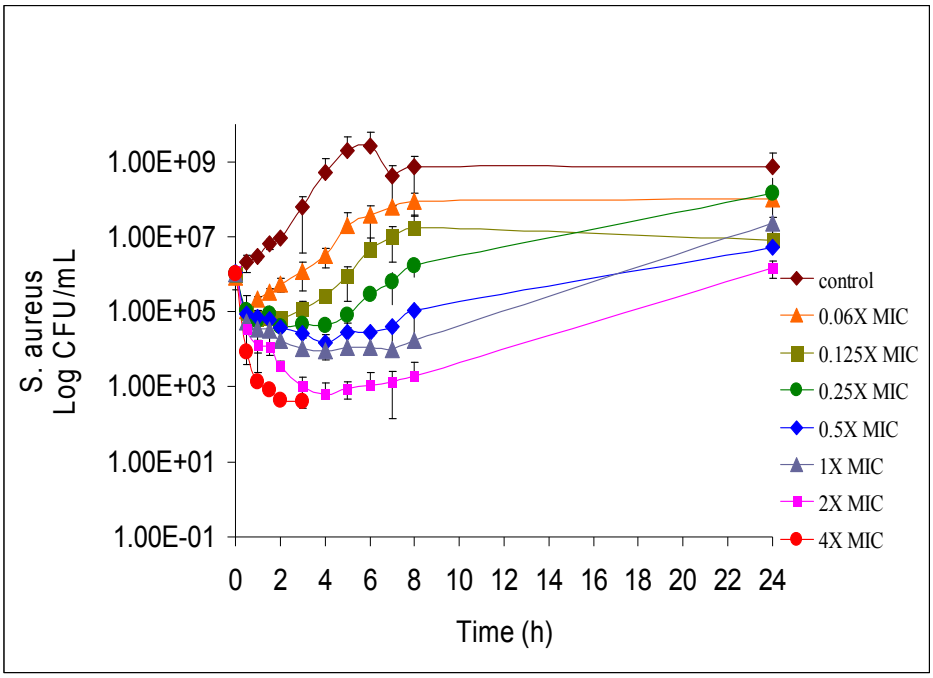


A

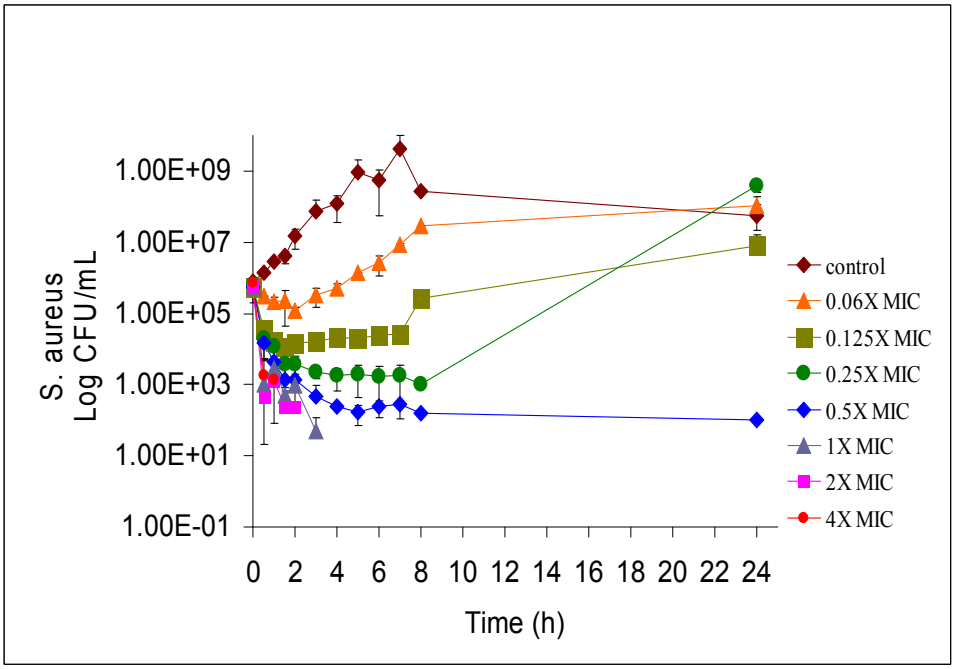


B

Figure 6-2. Time kill studies of MU1140 against *S. pneumoniae* in the absence and presence of 50% human serum, panels A and B, respectively.

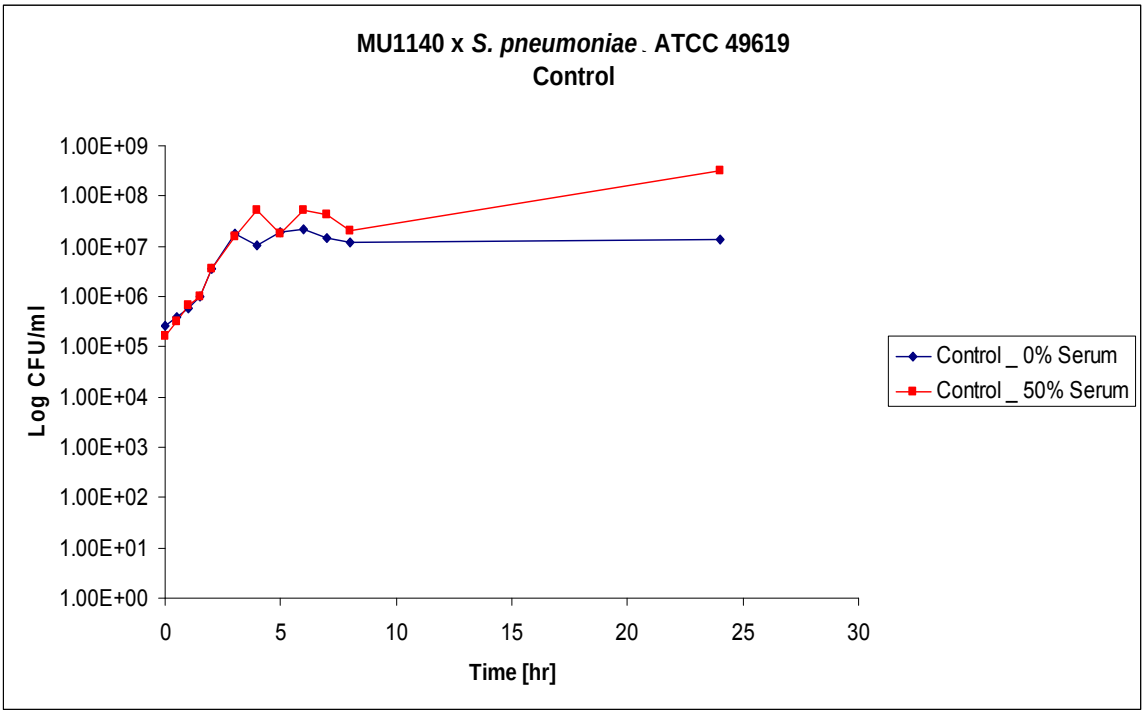


A

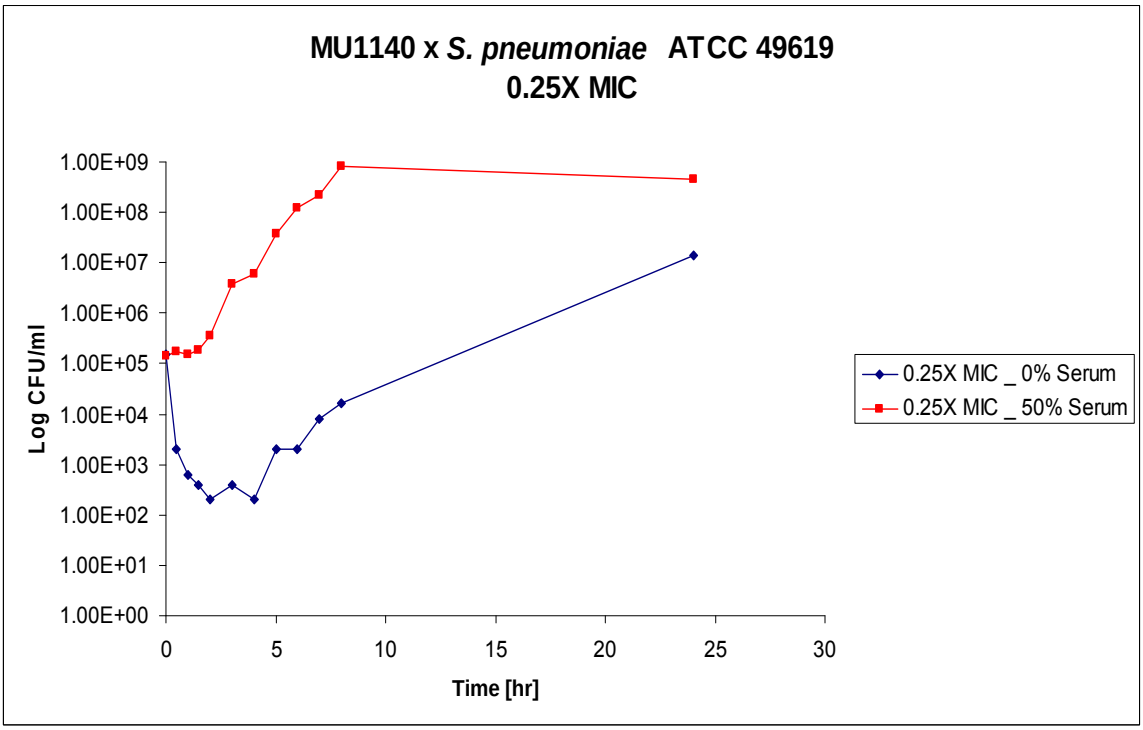


B

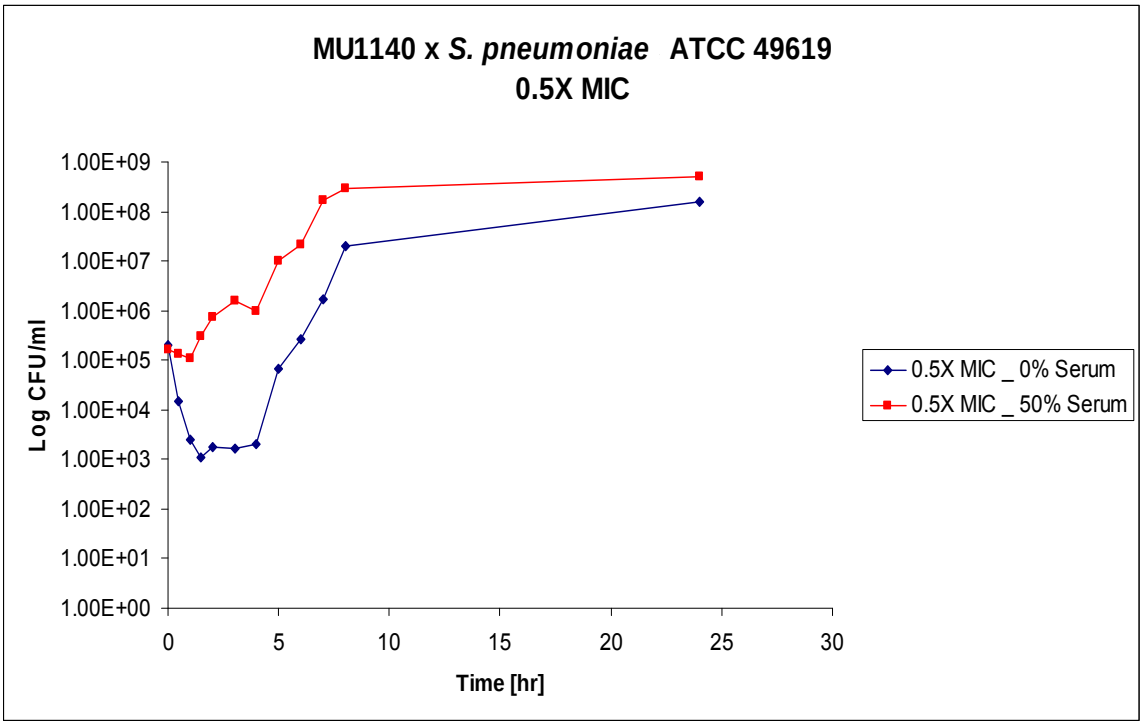
Figure 6-2. Time kill studies of MU1140 against *S. aureus* in the absence and presence of 50% human serum, panels A and B, respectively.


MU1140 x S. pneumoniae ATCC 49619
Control
Log CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
Time [hr]
Control _ 0% Serum
Control _ 50% Serum

A


MU1140 x S. pneumoniae ATCC 49619
0.25X MIC
Log CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
Time [hr]
0.25X MIC _ 0% Serum
0.25X MIC _ 50% Serum

B


MU1140 x S. pneumoniae ATCC 49619
0.5X MIC
Log CFU/ml
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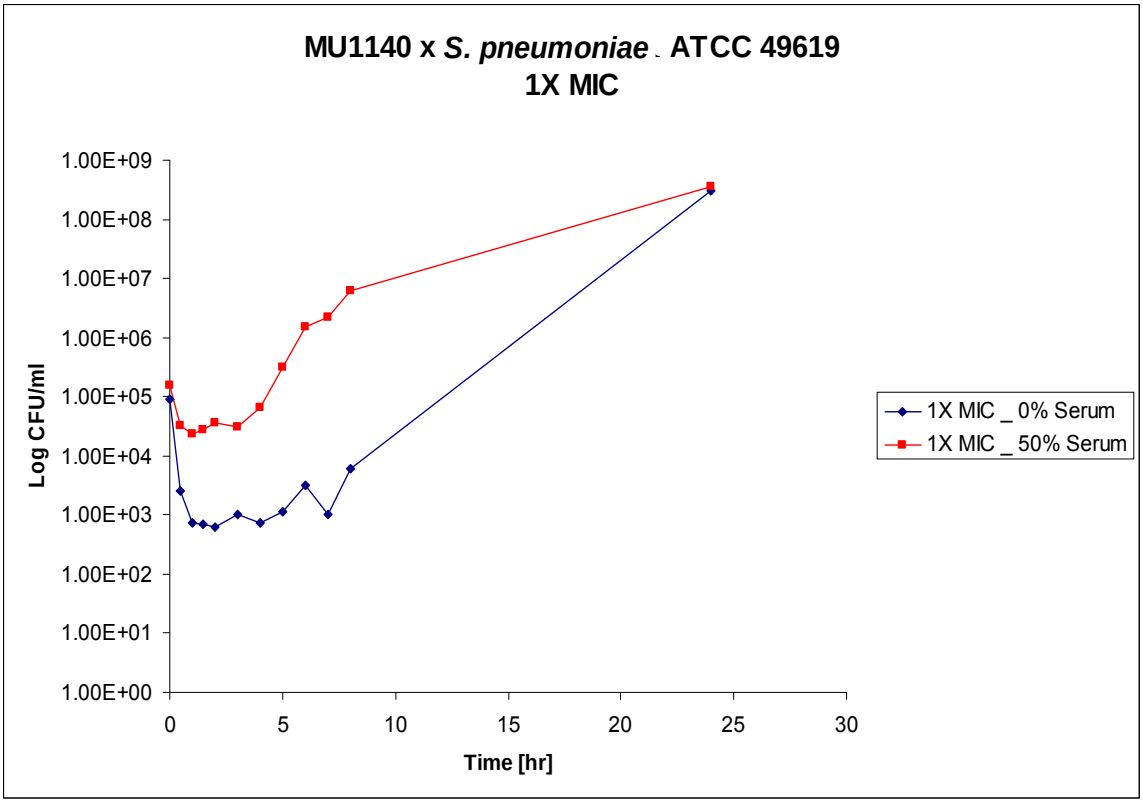
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
Time [hr]
0.5X MIC _ 0% Serum
0.5X MIC _ 50% Serum

C


MU1140 x S. pneumoniae ATCC 49619
1X MIC
Log CFU/ml
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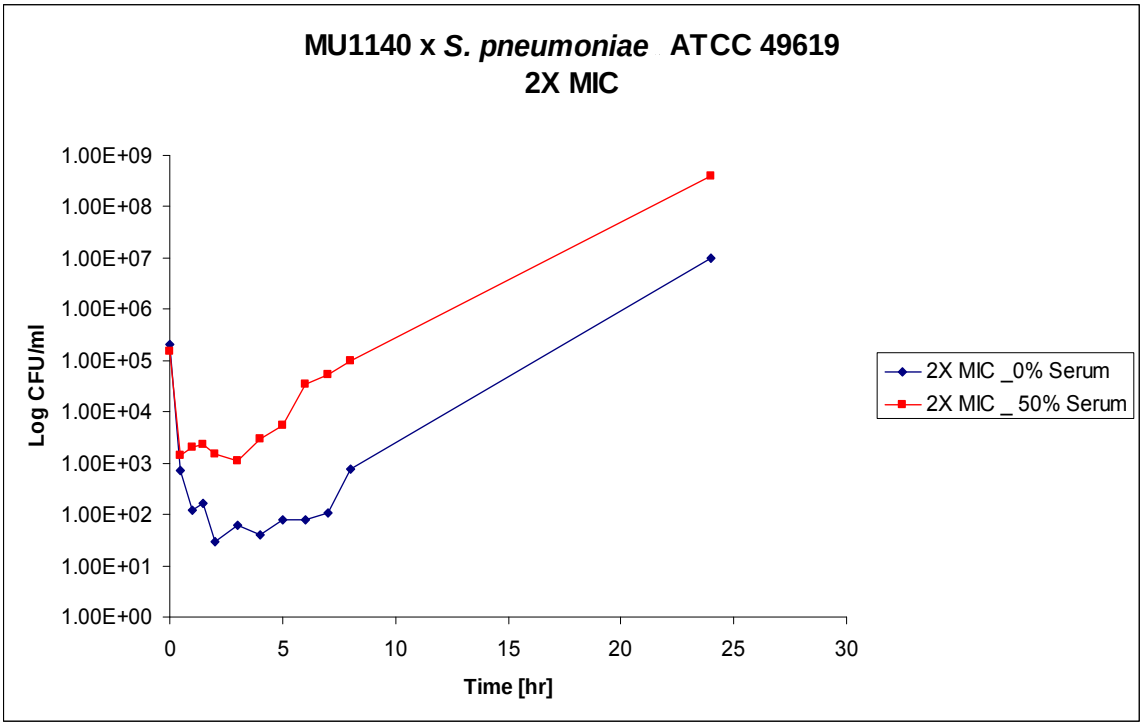
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
Time [hr]
1X MIC _ 0% Serum
1X MIC _ 50% Serum

D


MU1140 x S. pneumoniae ATCC 49619
2X MIC
Log CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
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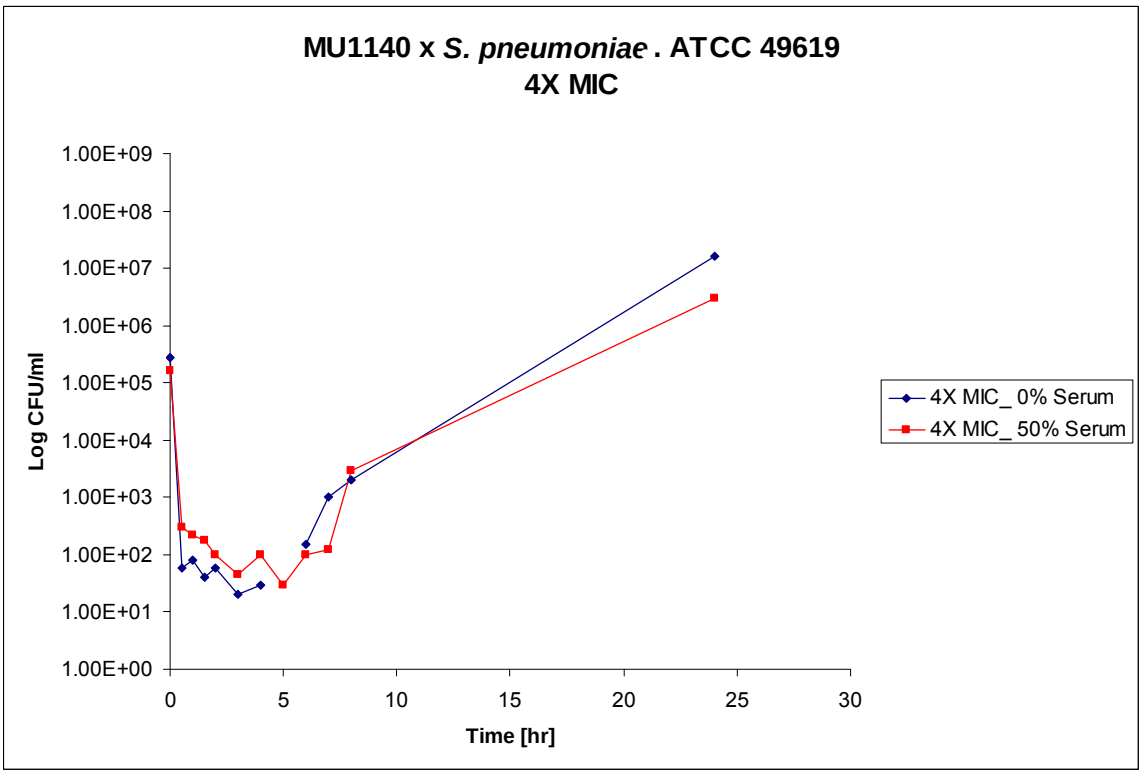
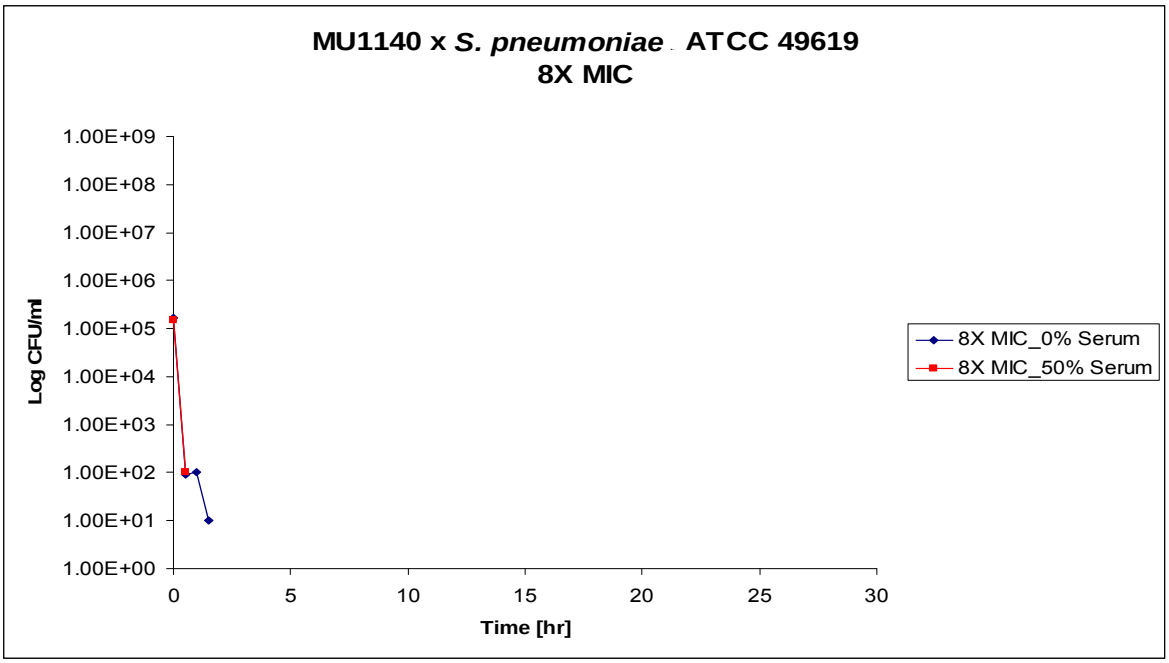
0
5
10
15
20
25
30
Time [hr]
2X MIC _0% Serum
2X MIC _ 50% Serum

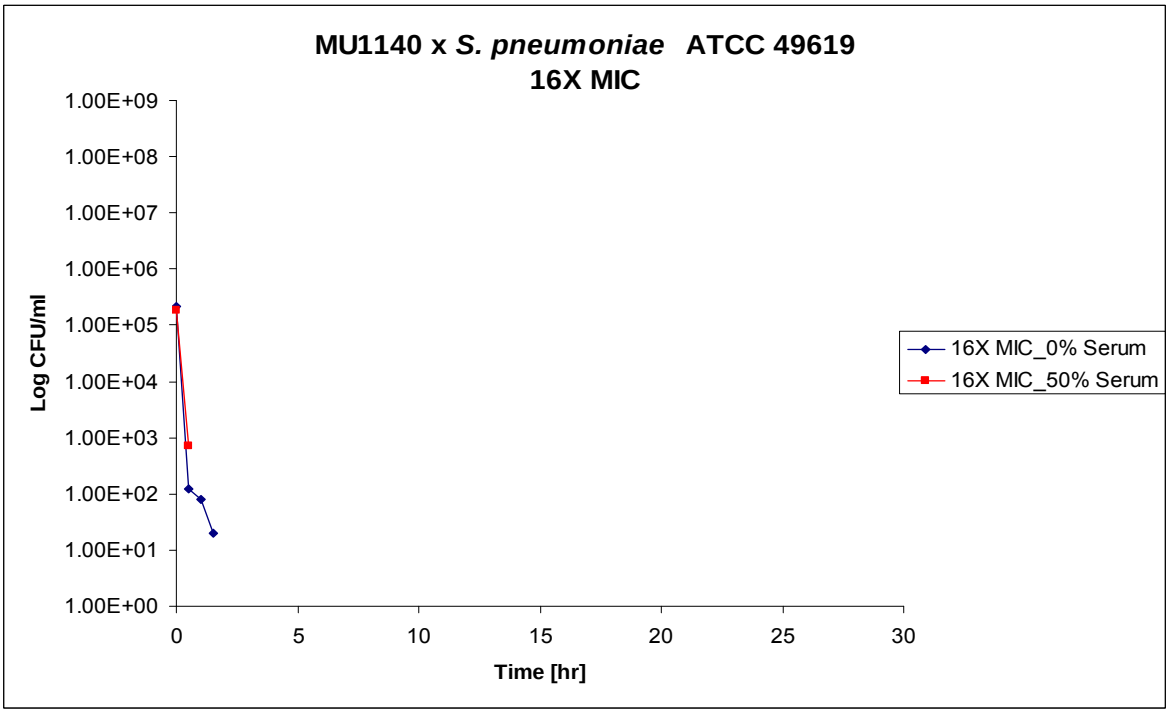
E


MU1140 x S. pneumoniae . ATCC 49619
4X MIC
Log CFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
5
10
15
20
25
30
Time [hr]
4X MIC_ 0% Serum
4X MIC_ 50% Serum

F

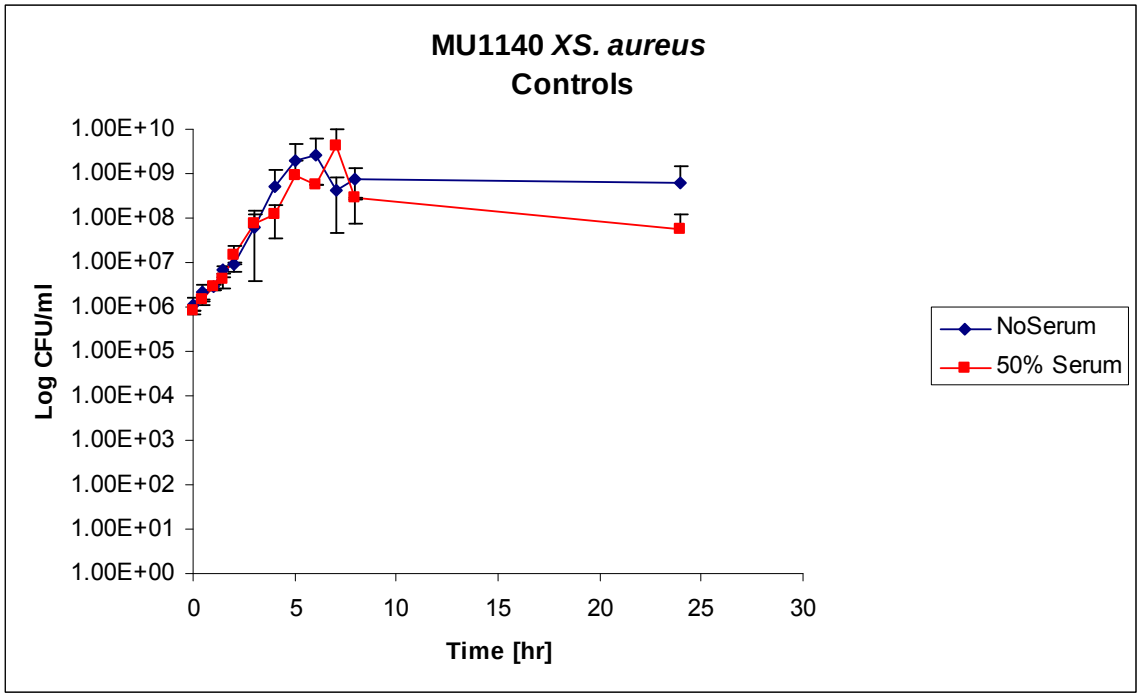


G

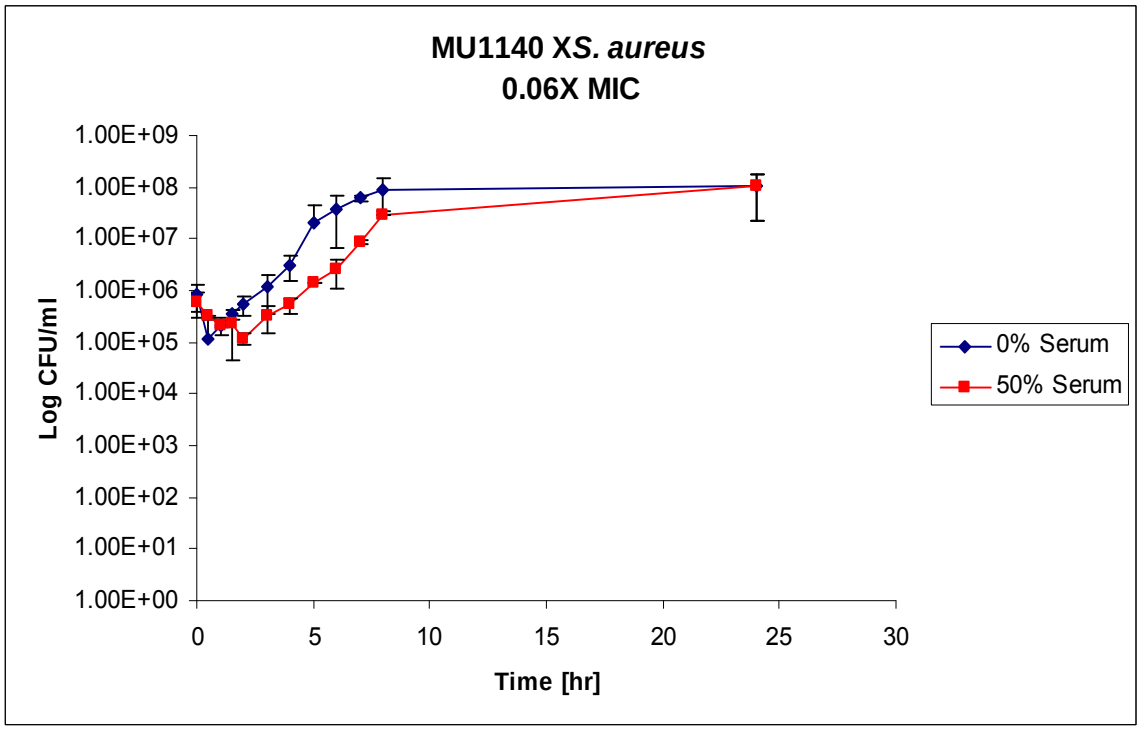


H

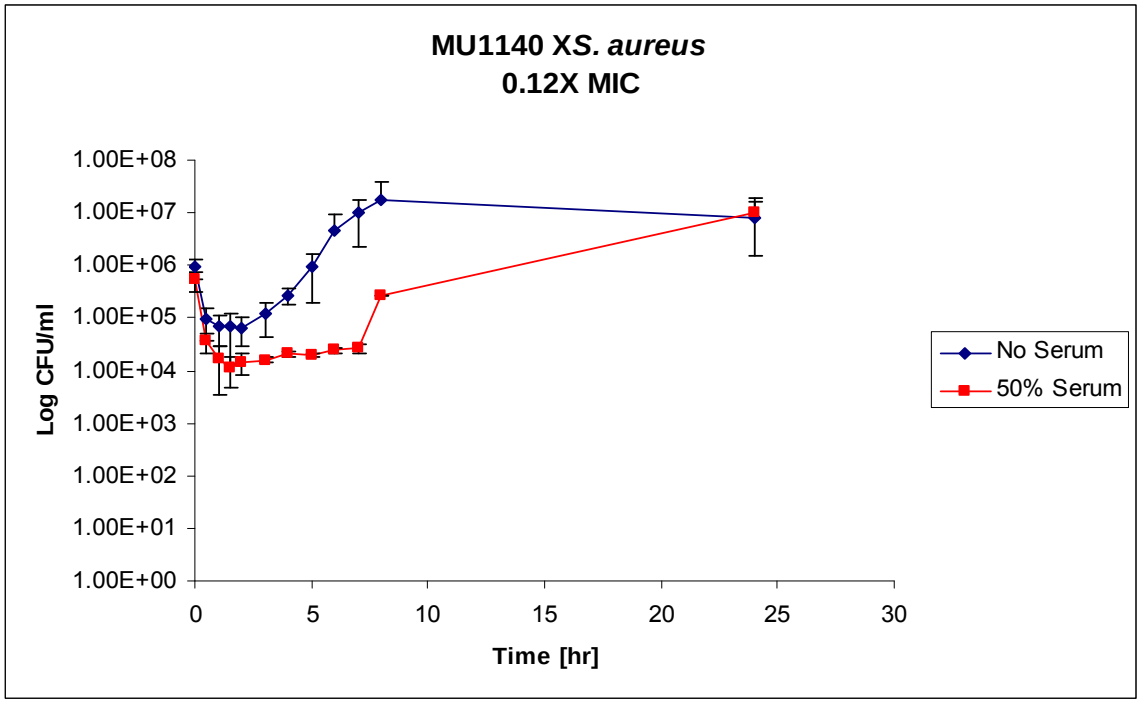
Figure 6-3. Side-by-side plot of *S. pneumoniae* viable cell counts in the presence of MU1140 and the presence and absence of human serum. MU1140 concentrations varied from 0.25-16 times MIC.


MU1140 XS. aureus
Controls
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
NoSerum
50% Serum

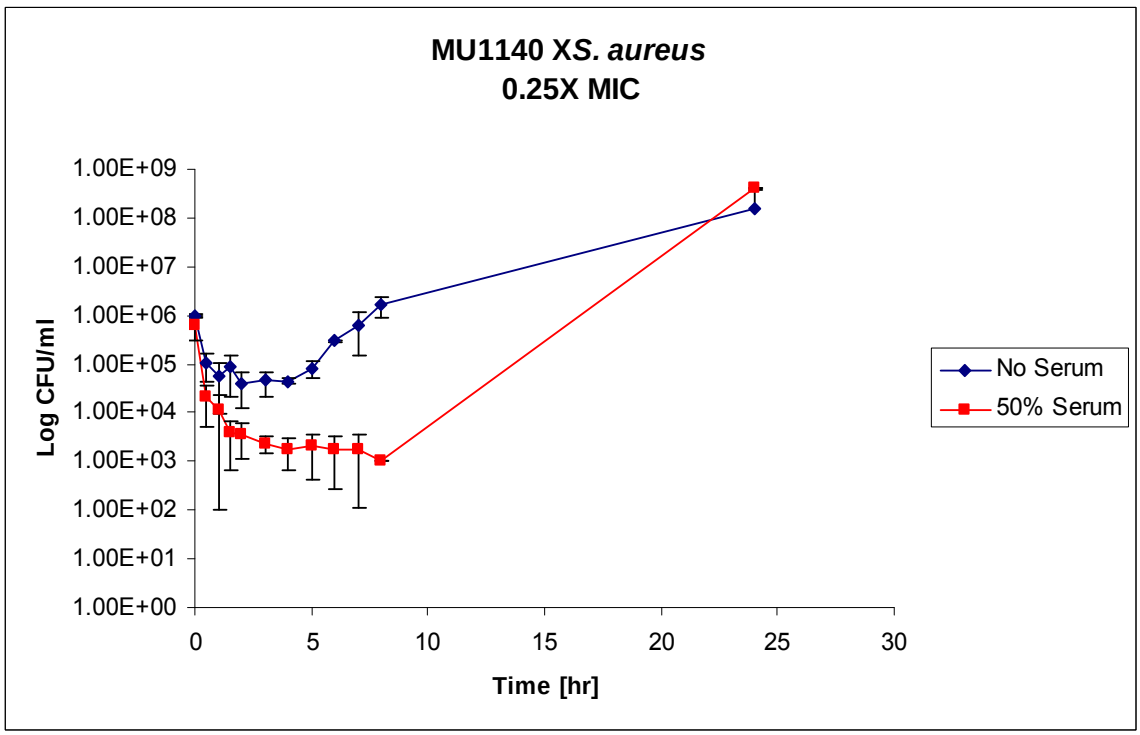
A


MU1140 XS. aureus
0.06X MIC
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
0% Serum
50% Serum

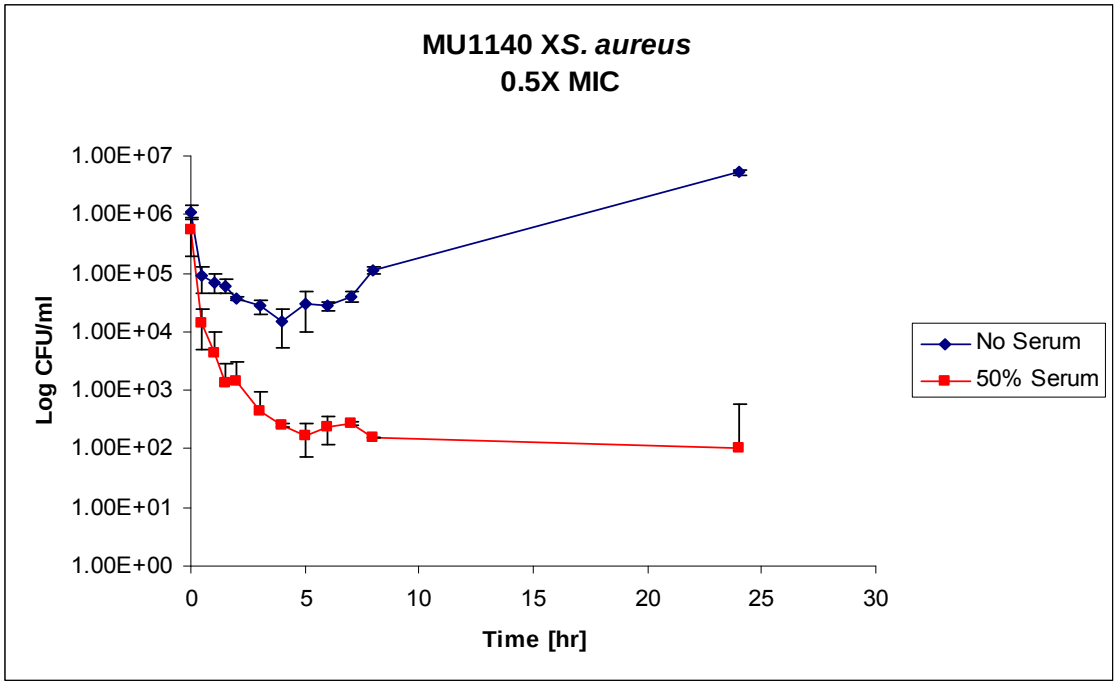
B


MU1140 XS. aureus
0.12X MIC
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
No Serum
50% Serum

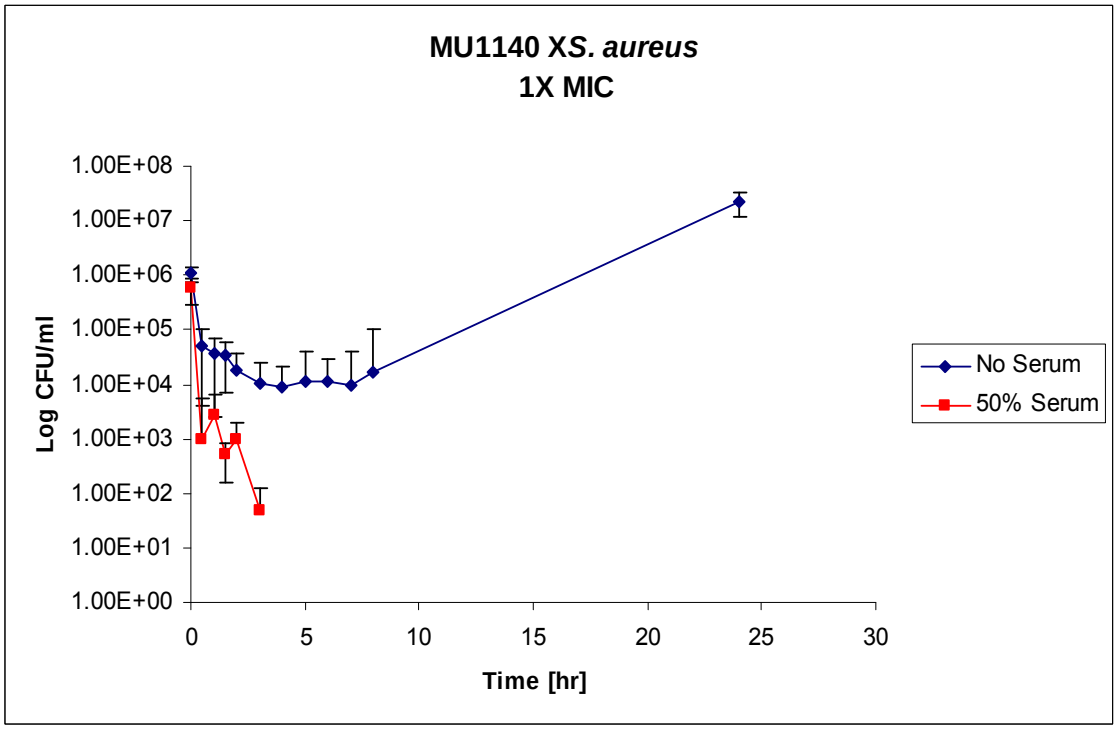
C


MU1140 XS. aureus
0.25X MIC
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
No Serum
50% Serum

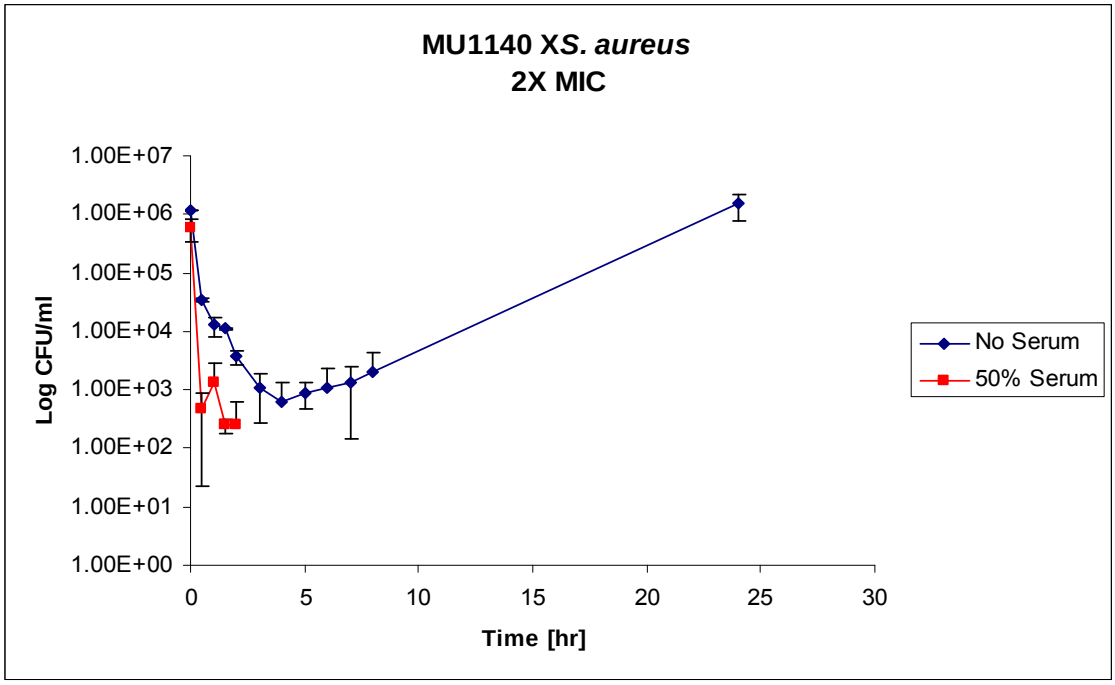
D


MU1140 XS. aureus
0.5X MIC
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
No Serum
50% Serum

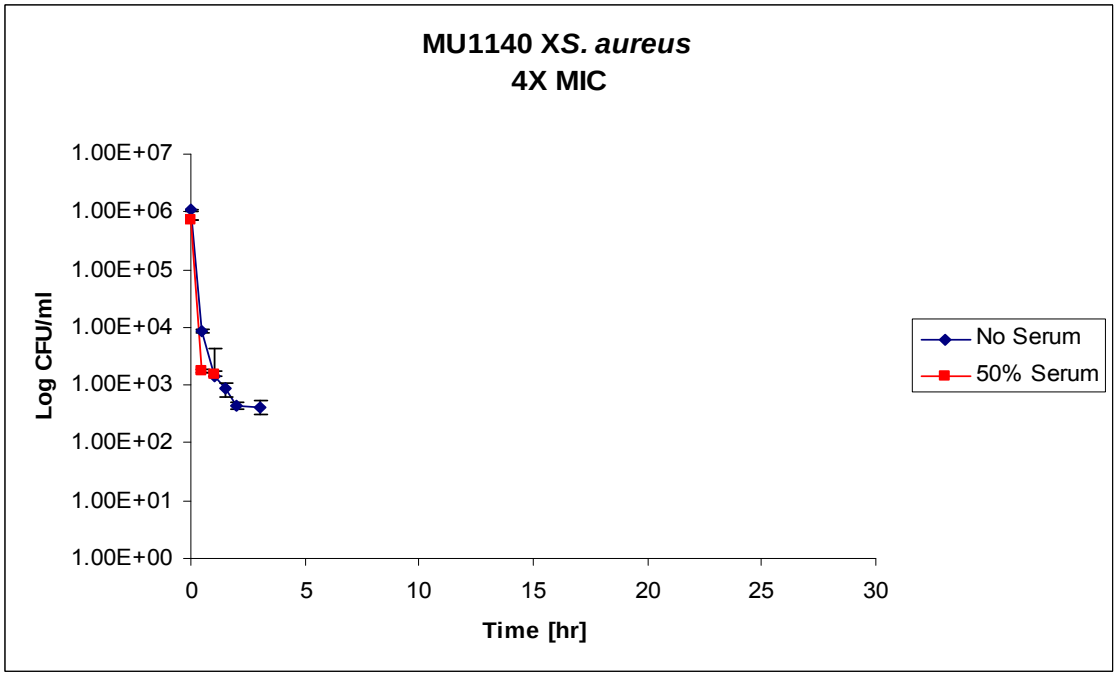
E


MU1140 XS. aureus
1X MIC
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Log CFU/ml
0
5
10
15
20
25
30
Time [hr]
No Serum
50% Serum

F

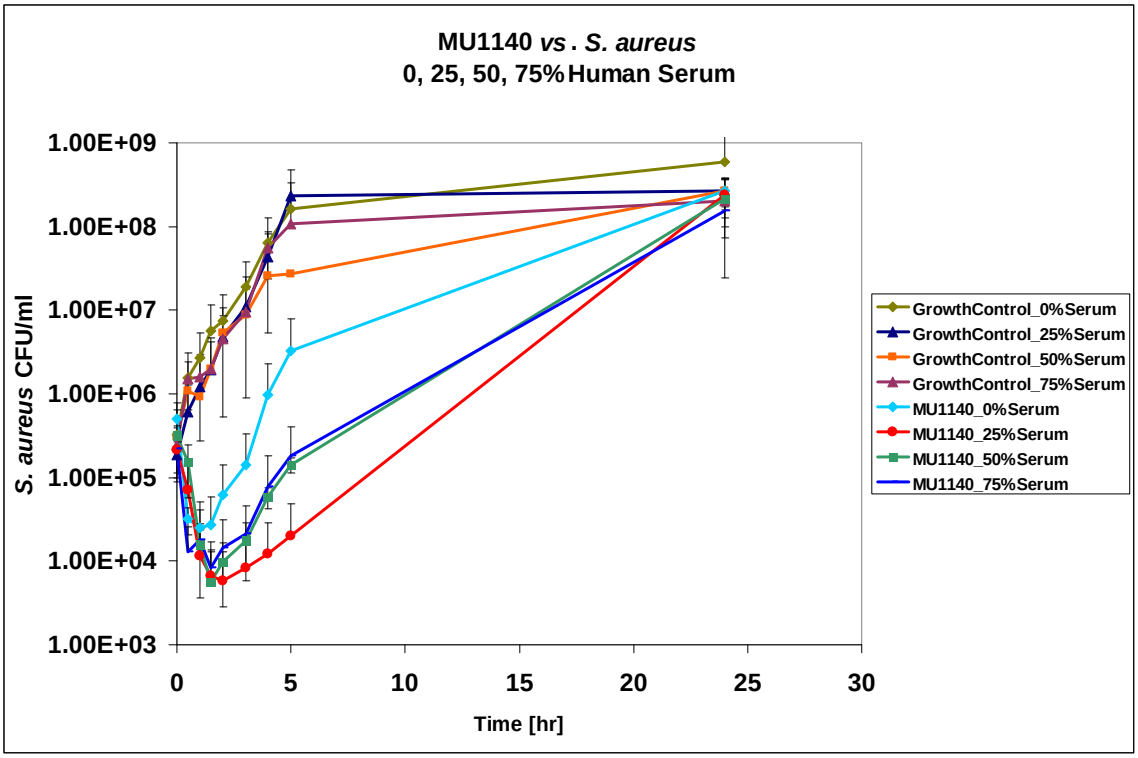


G

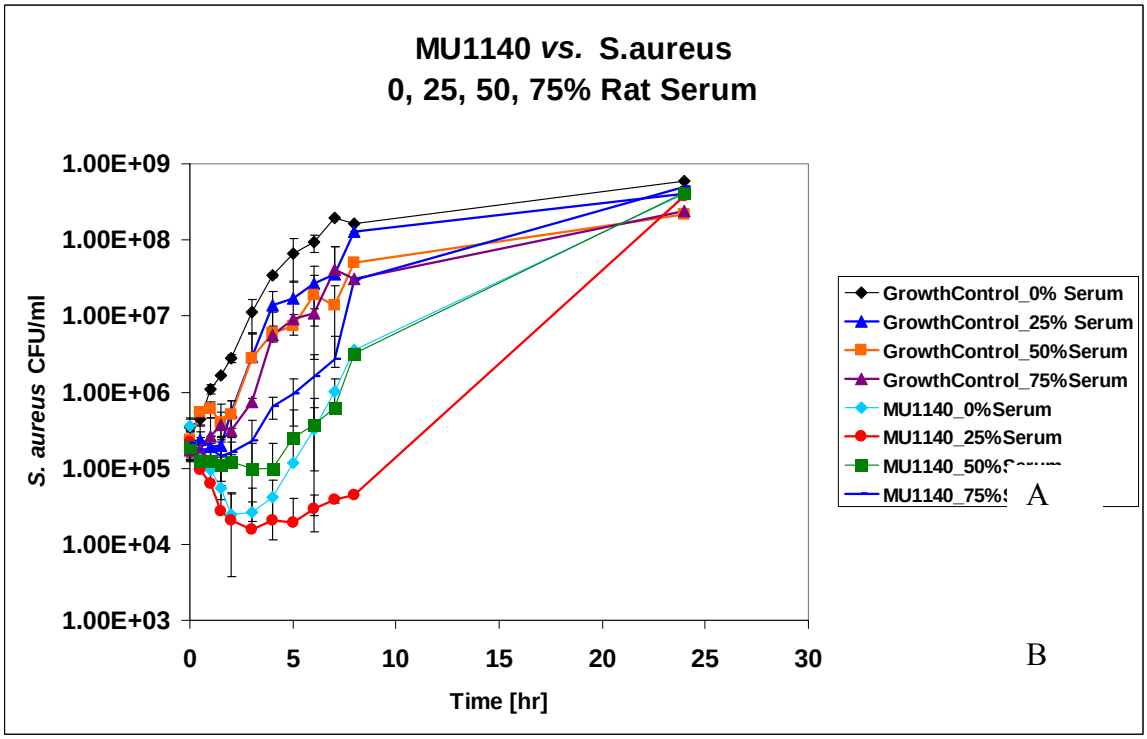


H

Figure 6-4. Side-by-side plot of *S. aureus* viable cell counts in the presence of MU1140 and the presence and absence of human serum. MU1140 concentrations varied from 0.06- 4 times MIC.



A



B

Figure 6-5. Time kill studies of MU1140 at 0.5 time MIC against *S. aureus* in the presence of various human or rat serum concentrations.

Tables

Table 6-1. MU 1140 MICs against *Streptococcus pneumoniae* (ATCC 49619) in the presence of 0, 25, and 50% inactivated human serum.

	MIC (μg/ml) 0% Serum	MIC (μg/ml) 25% Serum	MIC (μg/ml) 50% Serum
Mean (n=3)	0.8	1.6	3.2
SD	2.43 e-16	2.43 e-16	2.43 e-16

Table 6-2. MU 1140 MICs Multi Drug resistant *Staphylococcus aureus* in the presence of 0, 25, and 50% inactivated human serum

	MIC (μg/ml) 0% Serum	MIC (μg/ml) 25% Serum	MIC (μg/ml) 50% Serum
Mean (n=6)	4.8	1.6	1.6
SD	1.75	2.43 e-16	2.43 e-16

CHAPTER 7
CONCLUSIONS

As antibiotic resistant bacterial strains emerge, the need for new antibiotics with novel mechanisms of action is great. Appropriate dose design and proper use are the tools available to ensure success of therapy and efficacy maintenance of these newly developed antimicrobial agents. The work presented here is part of the preclinical development plan of the lantibiotic MU1140, an antibiotic indicated for the management and control of infectious diseases caused by Gram positive pathogens. Studies presented here are focused on the evaluation of the drug-like properties of MU1140 and its applicability to serve as a pharmaceutical agent for human use.

Production of MU1140 by fermentation and its purification process has been improved. The fermentation media composed of 5% yeast extract, 0.5% calcium chloride, and 4% glucose is the media mixture that triggered the highest production of MU1140. The purification method consisted of a precipitation step using ammonium sulfate followed by selective uptake of the activity in isopropanol and two separation steps using reversed phase chromatography where the activity is eluted with acetonitrile and methanol after the first and second separation steps, respectively. The yield of MU1140 according to this protocol is less than 1mg/liter which does not enable large scale commercialization of this antibiotic.

To study the *in vivo* behavior of MU1140, a bioanalytical method for its quantification in rat plasma was developed and validated. The developed method involves a simple and inexpensive liquid–liquid sample preparation followed by protein precipitation, filtration, LC-MS separation and detection procedure. This method was validated to be selective, accurate, precise, and sensitive, and the stability of MU1140 was not compromised during sample handling and processing.

The pharmacokinetics of MU1140 was investigated at a dose equivalent to 25 mg/kg rat body weight. The plasma concentration-time profile of MU1140 in rats declined biexponentially and was best fitted using an open two-compartment model with elimination from the central compartment and uniform weighing. MU1140's mean elimination half life was 1.7 ± 0.1 hrs. During the PK study, it was observed that rapid injection of MU1140 was not well tolerated. A hypersensitivity reaction, characterized by redness of the ears and paws and swelling, was observed within 5 minutes post-administration of the first dose. Subcutaneous administration of diphenhydramine 1 hour prior to MU1140 administration is sufficient to block most of these symptoms. In a clinical setting, pre-medication with DPA or administration of MU1140 as a slow infusion might be needed to block or avoid this reaction.

MU1140 *in vitro* pharmacodynamic investigation suggests a broad spectrum of activity against medically important Gram positive pathogens, including MRSA, VISA, VRSA, and VRE, which are responsible for most of infectious disease related deaths in US hospitals and worldwide. Time-kill studies reveal that MU1140 is bactericidal against *S. aureus* and *S. pneumoniae* in a concentration-independent manner, but bacteriostatic against *E. faecalis*. A PD model capable of predicting *S. aureus* concentrations resulting from different MU1140 static concentrations was developed. A PK/PD model for MU1140's activities was assembled which enables quantitative correlations of the antibiotic concentration and the bacterial concentration at any time point and thus a rational dosing regimen can be conceptualized.

MU1140 was found to highly bind (92.7% ± 2%) to serum proteins. *In vitro*, human or rat serum displayed a synergistic effect with MU1140 against *S. aureus*, where the addition of human/rat serum strongly augmented MU1140 bactericidal activity. *In vitro* efficacy warranted further investigation of the therapeutic potential of MU1140. Collectively these findings

illustrate the potential of MU1140 to serve as a therapeutic agent for the management of infections caused by multidrug resistant Gram positive bacteria.

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BIOGRAPHICAL SKETCH

Oliver Ghobrial developed a passion for life science at an early age. He earned his bachelor's degree in biological sciences and his master's degree in molecular and microbiology from the University of Central Florida.

Oliver moved to Gainesville, Florida to join Encor Biotechnology. He worked for Dr. Jerry Shaw where the focus of his project was recombinant DNA technology and antibodies production. Recently after, he joined the PhD program in the Department of Pharmaceutics at the University of Florida, working in the lab of Dr. Hartmut Derendorf. Oliver received his PhD in Pharmaceutics in summer 2008.

Oliver plans to work hard to improve human life on this planet.