18-β-GLYCYRRHETINIC ACID AND METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS:* FROM LYTIC ACTIVITY TO REDUCED PATHOGENESIS

by

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is Gram-positive pathogen known to cause severe disease in humans. MRSA's resistance to the β-lactam class of antibiotics makes it an increasing healthcare concern. Over the last two decades there has been a rise in the incidence of community-associated MRSA, specifically pulsed-field gel electrophoresis type USA300 (known to cause severe skin and soft tissue infections). The development of drug therapies against MRSA infections that do not induce resistance and have efficacy against MRSA is a pressing matter. In this study, we investigate the potential of two components in licorice extracts, Glycyrrhizic acid (GA) and 18-β-Glycyrrhetinic acid (GRA), as effective antimicrobials against MRSA. Using *in vitro* survival assays, we determined that GRA is directly bactericidal to MRSA. Using a murine model of skin and soft tissue infection, we discovered that topical treatment with GRA reduced severity of MRSA skin and soft tissue infections more rapidly than treatment with GA or a control. The increase in infection clearance was not due to a reduction in bacterial burden, but results indicate that GRA may decrease severity of the infection via an effect on the immune system. Treatment of an MRSA skin infection with GRA reduced expression of KC and G-CSF. To further investigate how GRA treatment led to a more rapid clearance of infection, we analyzed the expression of five *S. aureus* virulence genes (*saeR, hla,* RNAIII transcript, *mecA*, and *sbi*) after treatment with GRA or GA. GRA rapidly down-regulated four of the five virulence genes *in vitro* and all five virulence genes *in vivo* in the skin infection model. The data presented here shows that GRA is bactericidal, assists in decreasing the severity of MRSA infection via down-regulation of virulence genes, and can induce an altered immune response in the host.

INTRODUCTION

Background

Staphylococcus aureus (*S. aureus*) is a Gram-positive pathogen known to cause severe disease, ranging from skin and soft-tissue infections to bone, joint, and endovascular disease (47,47). *S. aureus* has been a leading cause of bacterial infection worldwide for decades (14,17,19,20) and has become known for how easily it incurs antibiotic resistance (13). After the introduction of penicillin in the 1940's, *S. aureus* quickly became resistant to the antibiotic through the incorporation of a plasmid encoding β-lactamase (19,39). Methicillin was introduced as an effective treatment against *S. aureus* in the late 1950's, and resistance to methicillin was seen as early as 1961 (6,19), giving rise to methicillin-resistant *Staphylococcus aureus* (MRSA). Methicillin resistance conferred resistance not only to methicillin, but to the entire class of β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems (13). Vancomycin has become the treatment of choice against MRSA, but due to the rigorous course of vancomycin prescribed to individuals afflicted with MRSA, vancomycin-resistant strains of *S. aureus* arose in the late 1990's (31,54,60,65,72,73,73). Also arising in the 1990's were strains of MRSA that are referred to as community-associated MRSA (CA-MRSA) (1,30). CA-MRSA strains can infect otherwise healthy people, are more virulent than other strains, and can spread more rapidly than other strains (30,66,69). *S. aureus* colonizes approximately 30% of the United States population (7,37), and approximately 5% of the population in the United States is colonized with MRSA (7,42,48). This fairly widespread colonization is important to the bacteria's ability to infect and spread among individuals (13). In the United States, MRSA is one of the leading causes of death by a bacterial pathogen (17,40). In 2005, there were 94,360 invasive MRSA infections in the United States, resulting in 18,650 deaths (40).

Antimicrobial Resistance

S. aureus has developed many ways to resist killing by antibiotics. There are two ways in which *S. aureus* can become resistant to β-lactam antibiotics: through production of a penicillinase encoded by *blaZ* (confers resistance to penicillin only) or through production of a β-lactam resistant penicillin-binding protein (PBP) (49). β-lactam antibiotics target the transpeptidase-active sites of PBPs, preventing them from completing peptidoglycan cell wall synthesis (24,49) The β-lactam resistant PBP, known as PBP2a, is encoded by the *mecA* gene, has an altered active site, and confers resistance to the entire class of β-lactam antibiotics (28,28,45,49,61,61). *S. aureus* strains containing the *mecA* gene are classified as MRSA (13). The *mecA* gene was shown to be part of a staphylococcal cassette chromosome (SCC*mec*) which is a mobile genetic element (13,34,38). Eight different major SCC*mec* complexes have been identified (SCC*mec*I-SCC*mec*VIII), with the *mecA* gene fully present in all eight and variations on the *mecR1* and *mecI* regulatory genes present in all eight, as well (13). CA-MRSA strains contain SCC*mec*IV or SCC*mec*V which contain a fully intact *mecA* and a truncated *mecR1*(13). SCC*mec*IV is the most widely distributed of the cassettes in *S. aureus* and isolates containing SCC*mec*IV grow faster than strains containing other allotypes (13). Glycopeptide antibiotics, such as vancomycin, bind to a terminus of cell wall peptidoglycan and prevent cross-linking (49). Glycopeptide resistance comes from one of two alterations: thickened or poorly cross-linked cell wall peptidoglycan that binds glycopeptides, or various genetic changes over time (i.e. altering the terminal end of peptidoglycan to prevent glycopeptide binding) (49). Resistance to some antibiotics can be regulated by various two-component systems of the bacteria, such as the MecR1-MecI (methicillin resistance) and VanRS (vancomycin resistance) systems (49). Various strains of MRSA have also developed resistance to other antimicrobials, such as the protein synthesis inhibiting ketolides, aminoglycosides, and tetracyclines, and the DNA replication inhibiting flouroquinolones (15,32,49).

Antimicrobial Targets

As published data shows, crude licorice extracts can directly alter the expression of drug resistance genes in MRSA (43). There are also additional genetic elements in MRSA that would make excellent targets for licorice treatment. Most antibiotics are directed at synthesis machinery and metabolism (49). However, *S. aureus* has the ability to modify its genome to avoid antimicrobial-mediated killing through antibacterial inactivation (i.e. penicillinase), drug efflux (i.e. tetracycline efflux pumps), and modification of drug target (i.e. *mecA* modification of cell wall elements) (49). A treatment designed to attack elements of the bacterial genome that are necessary for pathogenesis and survival is ideal due to the low possibility of these elements of the bacterial genome modifying for resistance. Some of these genome targets are the sixteen two-component systems (TCS) the bacteria uses to check its environment and regulate gene expression in response to environmental changes (56). The Agr (accessory gene regulator) system controls cell wall-associated proteins and exoproteins such as alpha-toxin, beta-hemolysin, leukotoxins, and toxic shock syndrome toxins through activation of the RNAIII regulatory gene element (11). The Agr system has been successfully targeted by antibodies to the *agr* auto-inducing peptide (AIP) and an RNAIII activating protein (RAP), resulting in a decrease in lethality of the bacteria (5,59). Blocking signaling in the Agr system causes a failure in Agr signaling and thus a lack of production of virulence factors, causing a reduced ability to infect a host (5,22,59). The SaeR/S (*S. aureus* exoprotein expression) TCS is important for dermonecrosis during MRSA skin infection and in the transcription of the *hla* gene, which encodes for alpha-hemolysin (51,56). Down-regulation or elimination of the SaeR/S TCS has been shown to ameliorate MRSA invasive and skin and soft tissue infections (56). Due to the importance of SaeR/S in disease caused by USA300, this TCS is another target for future therapeutics. Investigators recently began targeting virulence factors instead of genes essential for survival (such as cell wall particles), expecting that this method will decrease development of resistance and result in reduced severity and incidence of *S. aureus* disease (58). However, redundancy of virulence will require a treatment that is able to target multiple virulence determinants at once (58).

MRSA and the Immune System

The host’s immune response to MRSA is extremely important in the development and clearance of disease, and CA-MRSA strains have developed various methods of immune evasion. During MRSA skin and soft tissue infections, the cytokines IL-1α, IL-1β and IL-17, and γδ T cells and neutrophils are important in the clearance of infection

(18,50). Neutrophils are one of the first lines of defense against *S. aureus* infections. They are able to bind, ingest, and kill invading bacteria, utilizing reactive oxygen species (ROS), antimicrobial peptides (AMPs), and hydrogen peroxide produced within the cell (18,25). Opsonization of bacteria with complement other serum proteins can increase neutrophil activity against invading pathogens (18). CA-MRSA is able to block opsonization through production of a second immunoglobulin-binding protein (Sbi) and other complement inhibiting proteins (18,64). CA-MRSA strains can survive in a neutrophil due to production of pigment (protects against ROS), degradation of AMPs, and production of toxins such as leukocidins and hemolysins, some controlled by the SaeR/S TCS, which contribute to leukocyte lysis (18,71). Lysis of activated neutrophils, instead of apoptosis and ingestion by macrophages, releases neutrophil cytotoxic factors, bacterial toxins, and bacteria into the host, further contributing to disease (18,27,71). A moderation of the host immune response, specifically a reduction in neutrophils recruited to the site of infection, can lead to a more rapidly cleared infection, and less damage to the host (27). Thus, a therapeutic that is able to not only target virulence factors but also modulate the host immune system in a favorable way would be an ideal drug candidate.

18-β-Glycyrrhetinic Acid

Presently, there is significant effort to develop new antimicrobials against MRSA, however there are few candidate drugs currently undergoing trials that would be better than the available treatment options (10,21). Some of the new antimicrobial agents undergoing trials show no increase in the ability to mitigate infection, are not targeted against MRSA directly but are broad-spectrum antimicrobials, cannot be administered orally, and are likely to infer resistance over a short time period (13). Traditional antimicrobial therapy is not very effective for treating skin and soft tissue infections, creating a need for topical therapy apart from incision and drainage (13,46,52). Therefore, over the last decade there has been strong interest in the use of natural medicines as treatments for common diseases. Natural medicines are expected to alter immune function to prevent or clear infection (2,9,33,36,53,67,74) and also to alter the genetic expression of the pathogen (inhibition of replication, reduction in virulence, etc.) (29,43) One such natural medicine is a component of the licorice root plant *Glycyrrhiza glabra*, known as 18-β-Glycyrrhetinic acid (GRA) (57). Licorice has been used for centuries in Europe and Asia as treatment for ailments such as peptic ulcers and coughs (4). Interest in licorice extracts as modern medicines arose in the 1950's and continued through the 1970's, but this interest died down due to the emergence of some negative effects of habitual usage such as hypertension and electrolyte imbalance (57). However, renewed interest in the 1990's led to the discovery of licorice extracts, specifically GRA, as a treatment for many modern ailments and diseases (4). Various licorice extracts have been shown to be anti-inflammatory (3,12,36), anti-bacterial (43), anti-viral (16,67,68), and anti-tumor (35,63). Chloroform extracts from licorice root have been to shown to be not only directly bactericidal to *S. aureus* strains, including MRSA strains, but also to down-regulate genes necessary for methicillin resistance such as *mecA*, and *mecRI.* (43,55). GRA was shown to down-regulate expression of *hla* and *RNAIII* (44). Based on published data showing the effects of licorice compounds on *S. aureus,* and published data showing the synergism between Agr and SaeR/S, and our preliminary data on *SaeR* expression, we believe that GRA treatment will alter the gene expression on multiple virulence genes in MRSA (23). We hypothesize that GRA is able to reduce expression of multiple virulence factors, thus decreasing the severity of infections caused by MRSA and allowing the immune system to more readily clear an infection.

Licorice and the Immune System

Along with direct modification of pathogens, licorice extracts have also been shown to modify a host's immune system. GA has been shown to decrease MIP-1α and TNF-α production in macrophages (62) and decrease reactive oxygen species production in human PMNs (2), and GRA decreases TNF-α and IL-1α in macrophages (36) and inhibits C2 mediated lysis of erythrocytes (41). We anticipate that GRA will alter the immune system in a way that will contribute to clearance of MRSA and reduce the negative impact of inflammation (release of reactive oxygen species and proteases) to the host, leading to a reduction in disease severity. This could be very important in relation to MRSA infections due to MRSA's ability to evade and destroy parts of the host immune system, specifically polymorphonuclear leukocytes (PMNs), which contributes to host damage by MRSA virulence factors and cytotoxic compounds produced by the PMNs (70). PMNs are a first line of defense in *S. aureus* disease and are recruited to sites of infection by chemotactic signaling, and ingest (phagocytose) and kill bacteria through the production of reactive oxygen species and other bactericidal factors such as antimicrobial peptides (AMPs) and proteases (26). However, MRSA can survive phagocytosis and cause disease (26,27,70). MRSA has additional factors that help it to evade a host immune system, including inhibition of neutrophil chemotaxis through binding of PSGL-1 (8), resistance to antimicrobial peptides and reactive oxygen species through production of catalase, superoxide dismutase, and an AMP regulatory system, the ability to sequester host antibodies using protein A, the ability to cause lysis of host immune cells using leukocidins and hemolysins, and the inhibition of complement (18,26). There have been no studies on the effects of GRA on PMNs and their ability to assist in clearing an MRSA infection. Based on preliminary observations we hypothesize that GRA may increase the PMN's ability to kill the bacteria. Treating an MRSA infection with GRA should both reduce the bacteria's ability to survive and infect and increase the PMN's ability to kill the bacteria, without inducing any genetic changes to confer resistance.

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CHAPTER TWO

18-B-GLYCYRRHETINIC ACID INHIBITS MRSA SURVIVAL AND ATTENUATES VIRULENCE GENE EXPRESSION

Contribution of Authors and Co-Authors

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major source of infection in hospitals and in the community. Increasing antibiotic resistance in *Staphylococcus aureus* (*S. aureus*) strains has created a need for alternative therapies to treat disease. A compound from the licorice plant *Glycyrrhiza uralensis*, 18-β Glycyrrhetinic acid, has been shown to have antiviral, antitumor, and antibacterial activity. This investigation explores the *in vitro* and *in vivo* effects of this substance on MRSA pulsed-field gel electrophoresis (PFGE) type USA300. GRA exhibited bactericidal activity at concentrations exceeding 0.223 μM and, at less than lytic concentrations, inhibited growth over extended time periods. Upon exposure of *S. aureus* to sub-lytic concentrations of GRA, we observed a reduction in gene expression of key virulence factors, including *SaeRS* and *hla*. In murine models of skin and soft-tissue infection, topical GRA treatment significantly reduced skin lesion size and decreased the *in vivo* expression of *saeRS* and *hla* genes in *S. aureus*. Our investigation demonstrates at high concentrations GRA is bactericidal to MRSA and at sublethal doses can reduce virulence gene expression in *S. aureus* both *in vitro* and *in vivo.*

Introduction

Staphylococcus aureus is a Gram-positive bacterium that is known to cause severe disease world-wide (21,60). Drug-resistant *S. aureus* is a major public health concern (21) and methicillin-resistant *S. aureus* (MRSA) are resistant to killing by the entire class of β-lactam antibiotics (21) . MRSA infections, once restricted to the hospital setting, are now also common in the community (21). Besides being difficult to treat, infections caused by MRSA result in significant morbidity and mortality (64). MRSA is a leading cause of infections presenting to emergency departments across the United States, accounting for ~59% of skin and soft-tissue infections (68). In addition, mortality caused by MRSA in the United States exceed deaths caused by AIDS and tuberculosis combined (45).

Due to MRSA's resistance to β-lactam antibiotics, other courses of antibiotics are used to treat infections. However, the risk of increasing resistance is very high, and resistance is already being seen in respect to other antibiotics, such as clindamycin and erythromycin (21). Vancomycin remains the antibiotic used for definitive therapy (21). However, in 2002 the U.S. recorded its first case of vancomycin-resistant *S. aureus* (95). Thus, it is readily apparent that the continued use currently available antibiotics against this bacterium runs the risk of completely exhausting their usefulness (21). As an alternative to antibiotic use, there has been increased interest in finding natural compounds that have efficacy against bacterial infection (21,86).

One natural substance that has shown some antimicrobial promise is a triterpenoid saponin isolated from *Glycyrrhiza glabra*, a member of the licorice family (4). The isolated glycyrrhizic acid (GA) is considered metabolically inactive, and is hydrolyzed to 18-β-glycyrrhetinic acid (GRA) by commensal gut bacteria (38,46,79) at which point it can be absorbed into the bloodstream (46,79). GRA has various anti-tumor, anti-viral, anti-bacterial, and anti-inflammatory effects (4,25-27,55). GRA is able to restore the effects of β-lactam antibiotics against MRSA, inhibit replication of various viruses such as HIV, activate T cells, to inhibit reactive oxygen species generation, and increase macrophage-derived nitrogen oxide production (2,4,18,27,36,90). Collectively, GRA is efficacious against pathogens and appears to also modulate the host response.

In this study, we demonstrate GRA has potent bactericidal activity *in vitro* and show that GRA can reduce lesion size *in vivo* using a mouse model of *S. aureus* skin infection. Moreover, we demonstrate that GRA reduces expression of key virulence factors both *in vitro* and *in vivo* including *hla*, *saeRS*, *RNAIII*, *mecA* and *sbi*. These data show potential for GRA in treating MRSA skin infections and reveal that besides having antimicrobial capabilities, GRA reduces the virulence of MRSA by directly reducing expression of key virulence factors.

Results

GRA Attenuates Survival of MRSA *In Vitro*

Various crude licorice extracts have been shown to have bactericidal activity (27,36,56,72). We investigated the antimicrobial activities of pure Glycyrrhizic acid (GA) and 18-β- Glycyrrhetinic acid (GRA) extracts. Approximately 10^7 colony forming units (CFUs) of *S. aureus,* pulsed-field electrophoresis type USA300 strain LAC, were incubated for one hour in TSB with varied concentrations of GA or GRA. Bacteria re-suspended in DMSO and TSB were used as controls. Incubation with GA resulted in over 100% survival at all concentrations (Fig. 1A). Incubation with GRA resulted in less than 100% survival for all concentrations (Fig. 1B). Survival was dose-dependent, ranging from 0.023%±0.009 at 125 µg/ml to 67.13%±11.34 at 7.8125 µg/ml. Bacterial survival in control groups was over 350% (376.0%±35.86 for TSB+DMSO and 449.1%±60.35 for TSB only). These data suggest that GRA is bactericidal.

GRA Inhibits Growth of MRSA Over Time

Next we determined if GRA was bactericidal over a three hour time course. Bacteria were incubated in TSB alone, TSB with DMSO, or TSB containing varied concentrations of GA and GRA for three hours at 37^{o}C, with shaking. The optical density at 600 nm was measured every 15 minutes and at the end of the three hour time period, the bacteria were plated to determine surviving CFUs.

The survival over a three hour time period is shown in Figure 1 C-F, along with correlated peak optical density. As observed in the previous experiment, GA did not affect the survival of the bacteria (Fig. 1C). The survival of the bacteria in GA was equal to the survival of bacteria in media alone. When incubated with GA, the peak optical density after three hours was relatively the same across all GA concentrations and equal to the optical density of bacteria in media alone (Fig. 1E). GRA demonstrated dose dependent inhibition of growth (Fig. 1D). At the highest dose of 62.5µg/ml, the survival following the three hour incubation was 0.26%±0.13. At 31.25µg/ml the survival rate was 24.40%±13.41. The survival for both of these doses was statistically different from that of the bacteria grown in TSB only and TSB with DMSO. The bactericidal effect of GRA was lost at 15.625 µg/ml. At every concentration of GRA, there was a reduction in the optical density which correlates with the survival curve (Fig. 1F). These results are congruent with previously published results on the anti-bacterial effects of licorice extracts.

GRA Reduces Severity of MRSA Skin Infection

Using a mouse model of staphylococcal skin and soft tissue infection we determined the effect of topical treatment of GRA on the severity of mouse skin infections. Mice were infected subcutaneously with *S. aureus* (USA300). The injection site was treated daily with a topical application of 600 µg GA or GRA suspended in 5 µl DMSO or DMSO alone as a control. From day three to day seven, treatment with GRA significantly reduced the size of the abscess by 39.97%±5.53 compared to DMSO treatments when measured on days 3 and 7, while GA showed no activity (Fig. 2A).

Since GRA caused reduced bacterial survival *in vitro*, we performed experiments to determine if reduced bacterial survival following treatment with GRA occurred *in vivo*. One hour after infection, mice were treated with 600 µg GA or GRA suspended in DMSO, DMSO alone, or given no treatment. Twelve and twenty-four hours after infection mice were euthanized and the site of infection was excised and CFUs were enumerated. There was no significant difference in bacterial CFUs recovered between any of the other treatment groups at twelve hours or twenty-four hours (Fig. 2B).

GRA Modulates the Local Host Response to MRSA Infection

Previous studies have shown that GA has little effect on neutrophil function and the pro-inflammatory cytokines IL-1β and IL-6, but contributes to anti-inflammatory responses via inhibition of signaling pathways (such as the PI3K/Akt pathway) and inhibiting the induction of IL-12 and IFN-γ (2,83,87). GRA has been shown to inhibit the classical complement pathway, reduce IL-6 expression, and inhibit inflammation via the PI3K/Akt pathway (47,53). However, no studies have been published that explore the effects of licorice extracts on immune responses in the skin. Since treatment with GRA reduced the severity of infection but did not reduce bacterial burden we hypothesized that GRA could influence the host response. Following skin infection and treatment with GRA, GA, or DMSO alone we investigated the expression of thirteen cytokines via cytometric bead array including: IL-10, IL-17A, TNF, IFN-γ, IL-6, IL-4, IL-2, IL-1α, IL-1β, G-CSF, GM-CSF, KC, and MIP-1β. Of the thirteen cytokines investigated, only two demonstrated significant differences between GRA treated mice and control DMSO treated mice. At twelve hours post-inoculation, the level of KC in the tissue homogenate of the infected but untreated mice was significantly lower compared to the expression of KC in the infected and GRA treated mice (191,447±21386 pg/ml per g of tissue and 99301±5414 pg/ml per g of tissue, respectively) (Fig. 3A). At twenty-four hours post-treatment, the level of KC in the tissue homogenate of the uninfected and untreated mice was significantly lower than the level of KC in the uninfected but GRA treated mice (6572±1101 pg/ml per g of tissue and 2141±466.3 pg/ml per g of tissue, respectively) (Fig. 3B). At twenty-four hours post-inoculation, the expression of G-CSF in the infected but untreated mice was significantly increased compared to the level of G-CSF in the infected and GRA treated mice (278,877±46797 pg/ml per g tissue and 73083±13091 pg/ml per g of tissue, respectively) (Fig. 3C). These data suggest an anti-inflammatory effect of GRA during MRSA skin and soft tissue infection.

GRA Alters Gene Expression of MRSA *In Vitro*

Previous studies have shown the effects of various licorice extracts on the *in vitro* gene expression of MRSA, and showed changes in genes such as *mecA* and *hla* (56,57). To determine if GA or GRA had any influence on virulence factor expression we incubated USA300 with a sub-lethal dose of GRA or GA (52.5µg/ml) and measured transcript abundance of key *S. aureus* virulence factors *saeR, hla, RNAIII, sbi,* and *mecA* one hour post exposure to the licorice extracts. Transcript abundance of *saeR*, the regulatory gene component of a global virulence regulatory system SaeR/S, which has been shown to be very important in the development of staphylococcal skin lesions in mice (74) was down-regulated 14-fold versus control (Fig. 4). *Hla* which encodes alpha-toxin, a virulence factor responsible for dermo-necrosis in mouse skin infections (50) was down-regulated 11-fold compared to control, while *sbi*, a gene encoding an immunomodulatory protein important in antibody and complement evasion (85), was down-regulated 74-fold (Fig. 4). In addition, *mecA*, which encodes for altered penicillin-binding protein 2a to confer resistance to beta-lactam antibiotics, was down-regulated 18-fold compared to control (Fig. 4). The only gene up-regulated upon incubation with GRA was RNAIII transcript, the effector gene of the quorum-sensing Agr two-component regulatory system (over 46-fold increase) (Fig. 4). The reduction in expression of four major *S. aureus* virulence genes suggests that GRA may differentially regulate an even larger number of *S. aureus* genes.

GRA Alters Gene Expression of Key MRSA Virulence Factors *in vivo* During Murine Skin Infection

The severity of MRSA skin infection, based on the size of the abscesses, was decreased upon treatment with GRA (Fig. 2) and this reduction was not due to a significant decrease in bacterial burden. To further investigate why the severity of infection was reduced upon treatment with GRA, mice were infected subcutaneously with MRSA and then were treated with GRA or GA suspended in DMSO or DMSO alone as a control. At designated time points mice were euthanized, and bacterial mRNA was extracted from infected tissue and subjected to TaqMan RT-PCR with primers and probes specific for the same genes investigated *in vitro* (*saeR*, *hla, sbi*, *mecA*, and *RNAIII*).

Twelve hours post-infection, GRA did not influence MRSA gene expression of the selected targets relative to control, DMSO-treated mice (Fig. 5A). Down-regulation of all five genes occurred in GA treated mice (ranging from -12.656±18.318 for *RNAIII* to -66.402±69.429 for *Hla*) (Fig. 5A). However, at twenty-four hours post-infection, all five genes virulence genes investigated were down-regulated in the GRA treated mice (from -3.243±0.412 for *sbi* to -6.798±4.816 for *hla*) while little to no differential regulation was seen in the GA treated mice (all genes less than 1.5-fold change) (Fig. 5B). Seventy-two hours post-infection, *saeR* and *hla* were down-regulated in the GRA treated mice (-27.204±20.596 and -37.297±28.558, respectively) and slightly up-regulated in the GA treated mice (1.886±3.607 and 3.037±4.326, respectively) (Fig. 5C). At ninety-six hours post-infection, *saeR* and *hla* were still slightly down-regulated in the GRA treated mice (-3.016±2.280 and -6.538±4.590, respectively) as well as in GA treated mice (-2.973±3.334 and -3.676±3.907, respectively) (Fig. 5D). These data suggest that the reduction in MRSA skin infection severity may be due to a reduction in the virulence of USA300 upon treatment with GRA.

Discussion

In the current study, we found that GRA inhibits MRSA survival in a dose-dependent manner, as determined by colony counts and optical density (Fig. 1B, D, F). The data show a dose-dependent bactericidal effect over both short and extended time courses, when grown in rich and basic media. GA, another licorice root extract, showed no bactericidal activity and actually increased the survival of MRSA relative to controls (Fig. 1A, C, E). These data in part support previously published work showing the bactericidal effects of various crude licorice extracts, such as hexane fractionated licorice root (27,37,56,72). These data also are in contrast to a recently published study showing no antibacterial effect of GRA against various strains of *S. aureus* (57). While the basis for these differences currently is not clear, they could be due to the variation in bacterial strains used or variation in the preparation and delivery of the licorice extract.

The increased prevalence of skin and soft tissue infections (SSTIs) caused by MRSA along with the increased resistance to antibiotics, an enhancement of virulence, and the lack of a superior treatment for SSTIs caused by MRSA, demonstrates a need for the development of new therapies (23,45,66) PFGE type USA300 is known to cause severe SSTIs (45,68,73). Although we did not see a significant reduction in bacterial survival in the wound, there was a significant increase in the resolution of the wound when treated with GRA (Fig. 2A, B). During our investigation, we considered additional methods of topical treatment in order to boost the efficacy of our treatment. We were not able to develop an alternative treatment method that was satisfactory, however we are still investigating additional treatment approaches. An option that results in longer contact between the treatment and the site of infection could result in an increase in abscess healing and may reach bactericidal levels. GA treatment resulted in a slight decrease in abscess size. This may result from GA being transported through the cardiovascular system to the liver for clearance, where it may have entered the gut, been hydrolyzed into GRA, and circulated back through the cardiovascular system to the site of infection (79).

Another consideration to take into account when exploring treatment for SSTIs caused by MRSA is the immune response generated by the infection (65). The skin contains a vast array of cells that initiate an immediate innate immune response once the epidermal layer is breached. Antimicrobial peptides contained in the skin, along with keratinocytes, Langerhans cells, and many other immune cell types are set to respond immediately upon infection (65,77). Neutrophils are one of the most important immune cells in combating MRSA skin infection, using a variety of mechanisms to mediate MRSA killing (65). Neutrophils are recruited to the site of infection through cytokine and chemokine signaling. We investigated the effect of GRA treatment on the levels of various pro- and anti-inflammatory molecules in the skin of infected and uninfected mice. Upon treating the mice with GRA, we saw a decrease in two neutrophil recruitment factors, KC and G-CSF (Fig. 3A, B, C). Published studies have shown that a reduction, but not elimination, of neutrophils reduces bacterial burden and contributes to host survival (34). The reduction in neutrophil recruitment molecules may correlate with reduced neutrophil numbers at the site of infection which may account for less tissue damage and smaller lesion sizes. Future studies will determine if reduced KC and G-CSF correlate with reduced neutrophil numbers at the site of infection. This data indicates that GRA does have an immunomodulatory effect, however this effect seems slight and may not fully account for the reduction in abscess size after GRA treatment. The real magnitude of the immunomodulatory effect is not yet clear and is being further explored.

Since the immunomodulatory influence of GRA seemed subtle under the conditions of these experiments, we evaluated the virulence gene expression in *S. aureus* after incubation with the two compounds. We assayed five important *S. aureus* virulence genes. *SaeR*, the regulatory gene of the SaeR/S two-component system, is important for the development of an abscess in a murine skin model of infection and regulates the production of various virulence factors including hemolysins, DNase, and coagulase (30,67,74). *Hla*, alpha-hemolysin, is regulated by the SaeR/S and Agr two-component systems and has been shown to be an important factor in the development of CA-MRSA skin infections (29,50,74,96). The Agr two-component system is known for its role in quorum sensing but also plays a role in regulating virulence factors through the functions of the RNAIII transcript (69,97). The Agr system has been shown to be important in MRSA pathogenesis, and also in the regulation of methicillin resistance through the gene *mecA* (17). *MecA* encodes for an altered penicillin-binding protein 2 (PBP2a) which confers resistance to methicillin and the β-lactam class of antibiotics and is considered a target for antimicrobial therapies (14,63,86). *Sbi*, the second immunoglobulin-binding protein of *Staphylococcus aureus*, is a complement inhibitor, inhibits neutrophil phagocytosis, and has been shown to be regulated by the SaeR/S two-component system (74,85,99).

We saw a decrease in the expression of *saeR*, *hla*, *mecA*, and *sbi* after incubating *S. aureus* with GRA *in vitro* (Fig. 4). The decrease in *saeR* expression partially explains the down-regulation of *hla* and *sbi* since these two genes are under strong transcriptional control of the SaeR/S regulatory system (74). The down-regulation of *mecA* expression has been shown in published studies and may contribute to an increase in susceptibility to β-lactam antibiotics, leading to another avenue for treating MRSA infections (56). Exposure of MRSA to fractionated licorice root extracts can increase oxacillin susceptibility, which may stem from the reduction in *mecA* transcription (37). The up-regulation of RNAIII transcript could result from the bacteria sensing low numbers and thus turning on the Agr system in order to increase their rate of growth. The up-regulation of *RNAIII* contradicts recent findings that show down-regulation of RNAIII transcript after MRSA incubation with GRA, but this contradiction could be due to the variation in technique, strains used, and formulation of GRA (57).

The same trend of gene expression was seen *in vivo* after treatment with GRA, however RNAIII transcript was down-regulated at similar levels to the other four genes assayed twenty-four hours after initial treatment supporting observations made by others (Fig. 5A-D) (57). The down-regulation of these virulence genes correlates to the decrease in abscess size after GRA treatment.

In the current study we report the effect of GA and GRA on the survival and pathogenicity of MRSA PFGE Type USA300. We hypothesize that treatment with GRA would have an effect on MRSA in additional infection models such as sepsis, peritonitis, and endocarditis, leading to a more rapid clearance of bacteria and a reduction in the severity of disease through a combination of direct effect and modulation of both the host and pathogen. This study provides a foundation for future work to determine the effects of GRA and GA on additional infection models.

Materials and Methods

Bacterial Strains and Culture.

S. aureus cultures were grown in tryptic soy broth (TSB) containing 0.5% glucose. Cultures were inoculated from overnight growth at a dilution of 1/200 and grown at 37^{o}C with shaking at 250 rpm. For all studies, cultures were grown to mid-exponential phase (OD_{600} = 1.5) for use. Two strains were used for the studies: pulse-field gel electrophoresis types USA300 (LAC) and USA400 (MW2). For *in vitro* studies, cultures were washed and re-suspended in TSB or RPMI 1640 medium (Cellgro) containing 10 mM HEPES to 10^{8}/ml. For *in vivo* studies, cultures were washed and re-suspended in sterile DPBS (Cellgro).

Glycyrrhizic Acid and 18-β-Glycyrrhetinic Acid Preparation.

Stocks of GA (Fluka Analytical) and GRA (Aldrich) were suspended in 100% dimethyl sulphoxide (DMSO, Sigma). The stocks were frozen in aliquots of 25 µl at 25 mg/ml. For use, the frozen stock was diluted to 1.25 mg/ml in appropriate media. For *in vivo* studies, GA and GRA were prepared in DMSO at 60 mg/ml and 120 mg/ml.

Bactericidal Assays to Determine Effects of GA and GRA on Bacterial Growth.

Bacteria (~10^5) were re-suspended in RPMI or TSB with varied concentrations of GA and GRA, diluted from frozen stock in RPMI or TSB, in a 96-well tissue culture plate. The plate was incubated at 37°C for 1 hour and *S. aureus* was plated onto tryptic soy agar (TSA, EMD). Colony forming units (CFUs) were enumerated the next day and percent survival was calculated using the formula (CFU at *t*1/CFU at *t*0*100).

Bacterial Growth Kinetics.

Bacteria (~10^5) were re-suspended in TSB with varied concentrations of GA and GRA, in a 96-well tissue culture plate. Cultures were incubated on a Spectromax spectrometer for three or six hours at 37°C with agitation. The OD_{600} of cultures in each of the wells was recorded at 15 minute intervals using SoftMax Pro v5.4 software.

In Vitro Gene Expression.

Bacteria (~10^5) were re-suspended in TSB with 52.5 µg/ml of GA, GRA, or TSB alone. The concentration of GRA and GRA used was 52.5 µg/ml. Samples were incubated at 37°C for one hour and RNA was harvested and subjected to TaqMan real-time reverse transcriptase polymerase chain reaction (RT-PCR), as described previously (73,74,93). The relative quantification of genes was determined by change in expression of transcripts relative to expression of the housekeeping gene *gyrB* in untreated bacteria.

Data is expressed as change in transcript level after treatment with GA or GRA. The sequences of the primers and probes used for analysis are listed in Table 1.

Mouse Models of Infection.

All animal studies conformed to the National Institutes of Health guidelines and were approved by the Montana State University Institutional Animal Care and Use Committee. Female Crl;SKH1-hrBR hairless mice were purchased from Charles River Laboratories. Mice were inoculated subcutaneously with ~3.5*10^7 CFUs *S. aureus*, LAC strain, in sterile PBS. Mice were treated daily beginning on day three with topical application of 600 µg GA or GRA in DMSO or DMSO alone as a control. Abscess area was calculated daily. Reduction of infected area was calculated using the following formula:

$$\% \text{ Change} = (\text{Current size}-\text{Prior Day size})/\text{Prior Day size}$$

For gene expression and cytokine assays, mice were treated immediately after inoculation and then daily. Mice were then sacrificed at designated time points and abscesses were excised using a 9 mm punch and weighed. Excised material was used to determine CFUs, analyze gene expression (as described above), and to determine cytokine expression.

Cytokine Assays

A Cytometric Bead Array (CBA, BD Biosciences) was used to determine the concentration of IL-10, IL-17A, TNF, IFN-γ, IL-6, IL-4, and IL-2 and Bio-Plex Cytokine Assays (Bio-Rad) were used to determine the levels of IL-1A, IL-1B, G-CSF, GM-CSF, KC, MIP-1A, and MIP-1B from infected excised material. For skin infection studies, the abscesses were homogenized in sterile PBS and the supernatant from the resulting homogenate was subjected to a CBA or Bio-Plex to determine the presence of cytokines in the abscess. All assays were performed according to manufacturer instructions.

Statistical Analyses.

All data sets were analyzed using GraphPad Prism, version 5 for PC (GraphPad Software, San Diego, CA). All data sets were analyzed using one-way ANOVA with Tukey's post-test or two-tailed t-test as indicated.

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TABLE 1. List of probes and primers

Gene	Probe or Primer Sequence
***GyrB* probe**	5’-AATCGGTGGCGACTTTGATCTAGCGAAAG-3’
***GyrB* fwd**	5’-CAAATGATCACAGCTTTGGTACAG-3’
***GyrB* rvs**	5’-CGGCATCAGTCATAATGACGAT-3’
***SaeR* probe**	5’-ATTTACGCCTTAACTTTAGGTGCAGAT-3’
***SaeR* fwd**	5’-CTGCCAAAACACAAGAACATGATAC-3’
***SaeR* rvs**	5’-ATTTACGCCTTAACTTTAGGTGCAGAT-3’
***Hla* probe**	5’-ATGAATCCTGTCGCTAATGCCGCAGA-3’
***Hla* fwd**	5’-CAACAACACTATTGCTAGGTTCCATATT-3’
***Hla* rvs**	5’-CCTGTTTTTACTGTAGTATTGCTTCCA-3’
***RNAIII* probe**	5’-TGCACAAGATATCATTTCAACAATCAGTGACTTAGTAAAA-3’
***RNAIII* fwd**	5’-GTGATGGAAAATAGTTGATGAGTTGTTT-3’
***RNAIII* rvs**	5’-GAATTTGTTCACTGTGTCGATAATCC-3’
***MecA* probe**	5’-ATCTATAGCGCATTAGAAAA-3’
***MecA* fwd**	5’-ACTGATTAACCCAGTACAGATCCTTTC-3’
***MecA* rvs**	5’-TCCAAACTTTGTTTTTCGTGTCTTT-3’
***Sbi* probe**	5’-CAGGTAGCTTTATGGTTGCTACAAAAAT-3’
***Sbi* fwd**	5’-ATACATCAAAACATTACGCGAACAC-3’
***Sbi* rvs**	5’-CTGGGTTCTTGCTGTCTTTAAGTG-3’

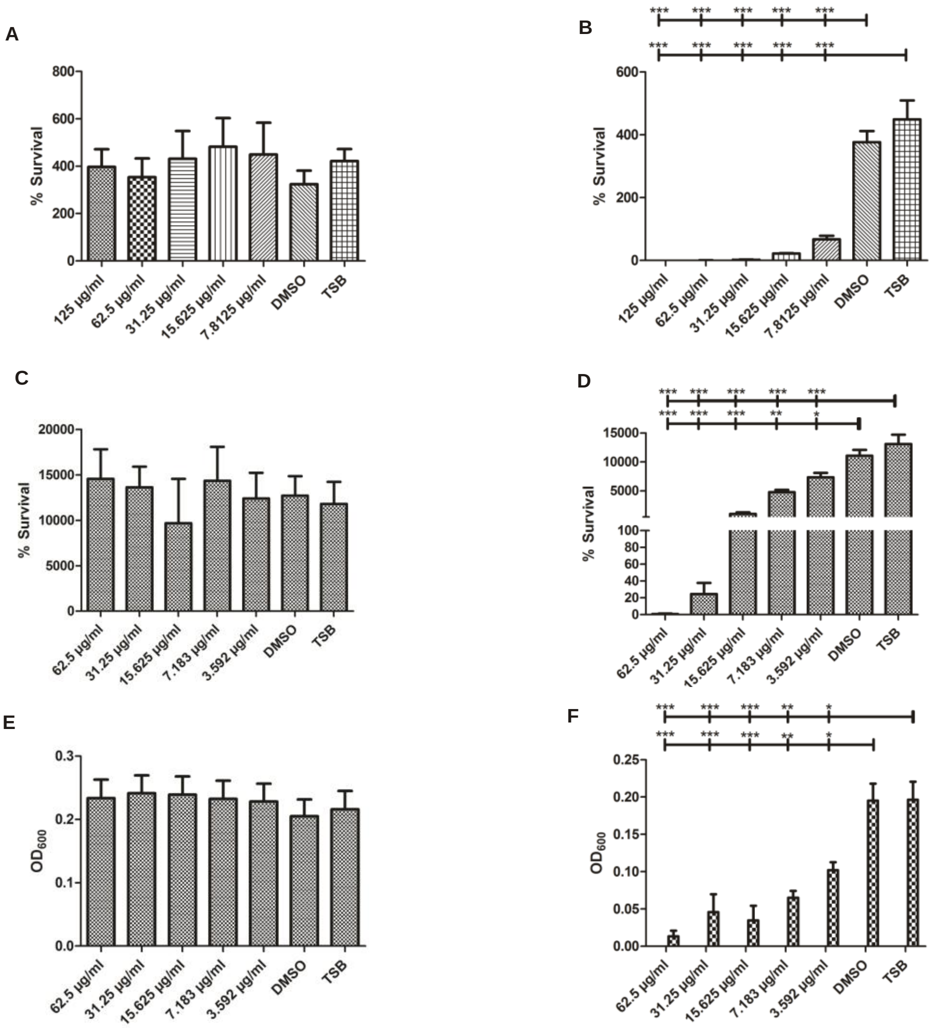

Fig. 1. GRA inhibits MRSA survival in a dose-dependent manner. Approximately 10^7 cfu LAC were incubated in media with varied concentrations of GA or GRA. (A) One-hour survival of LAC incubated in TSB with varied concentrations of GA (n=3). (B) One-hour survival of LAC incubated in TSB with varied concentrations of GRA (n=3). (C) Three-hour survival of LAC incubated, with shaking, in TSB with varied concentrations of GA (n=4). (D) Three-hour survival of LAC incubated, with shaking, in TSB with varied concentrations of GRA (n=4). (E) Peak OD600 of LAC incubated, with shaking, for three hours in TSB with varied concentrations of GA (n=4). (F) Peak OD600 of LAC incubated, with shaking, for three hours in TSB with varied concentrations of GRA (n=4). OD600, optical density measured at 600 nm. *P<0.05, **P<0.01, and ***P<0.001, as determined by ANOVA and Tukey’s post-test.

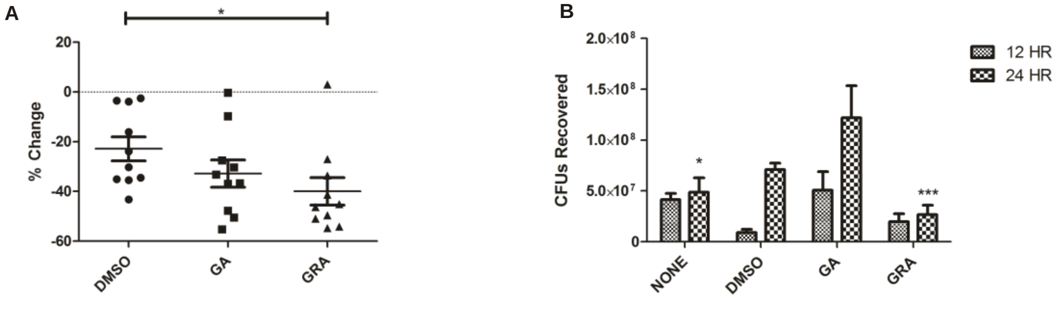

Fig. 2. GRA attenuates MRSA severity during skin infection. Mice were inoculated subcutaneously with MRSA (2×10^7). (A) Percent change in abscess size over four day period. Abscess size was measured daily prior to treatment with DMSO, GA, or GRA. (B) Survival of MRSA in murine skin at twelve and twenty-four hours post-inoculation. DMSO, GA, or GRA was applied topically one hour post-inoculation. A significant difference in CFUs was seen in untreated skin and GRA treated skin when compared to GA treated skin. *$P<0.05$, ***$P<0.001$ as determined by two-tailed t-test.

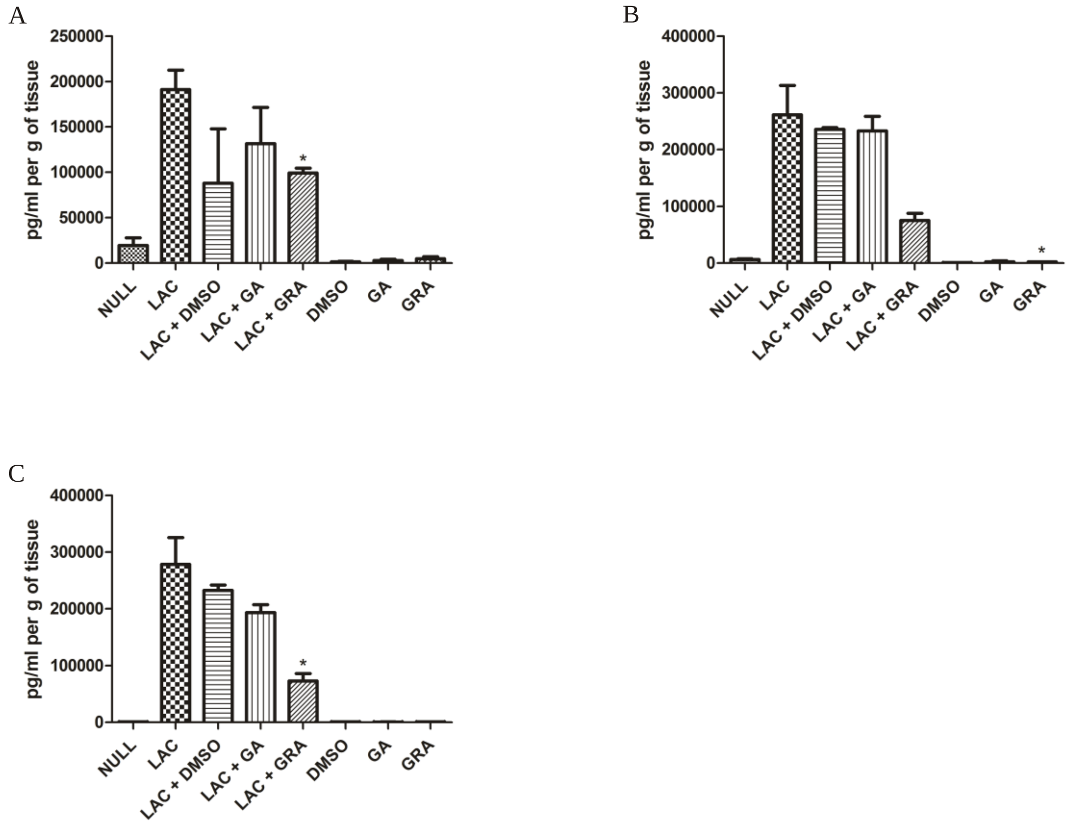

Fig. 3. GRA induces altered cytokine response in skin. (A) Concentration of KC at site of inoculation/treatment twelve hours post-inoculation. There is significantly less KC in infected and GRA treated (LAC+GRA) mice when compared to infected and untreated mice (LAC). (B) Concentration of KC at site of inoculation/treatment twenty-four hours post-inoculation. There is significantly less KC in uninfected and GRA treated mice (GRA) than uninfected and untreated mice (NULL) (C) Concentration of G-CSF at site of inoculation/treatment twenty-four hours post-inoculation. There is significantly less G-CSF in infected and GRA treated mice (LAC+GRA) when compared to infected and untreated mice (LAC). Results are from three mice. *P<0.05 as determine by two-tailed t-test.

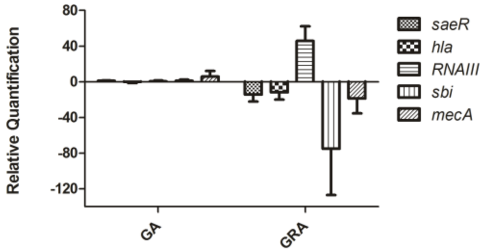

Fig. 4. GRA alters *S. aureus* virulence gene expression *in vitro*. Relative quantification of five *S. aureus* virulence genes after one hour incubation with GA or GRA. Data is normalized to *gyrB* expression and fold-change is relative to *S. aureus* incubated in media alone.

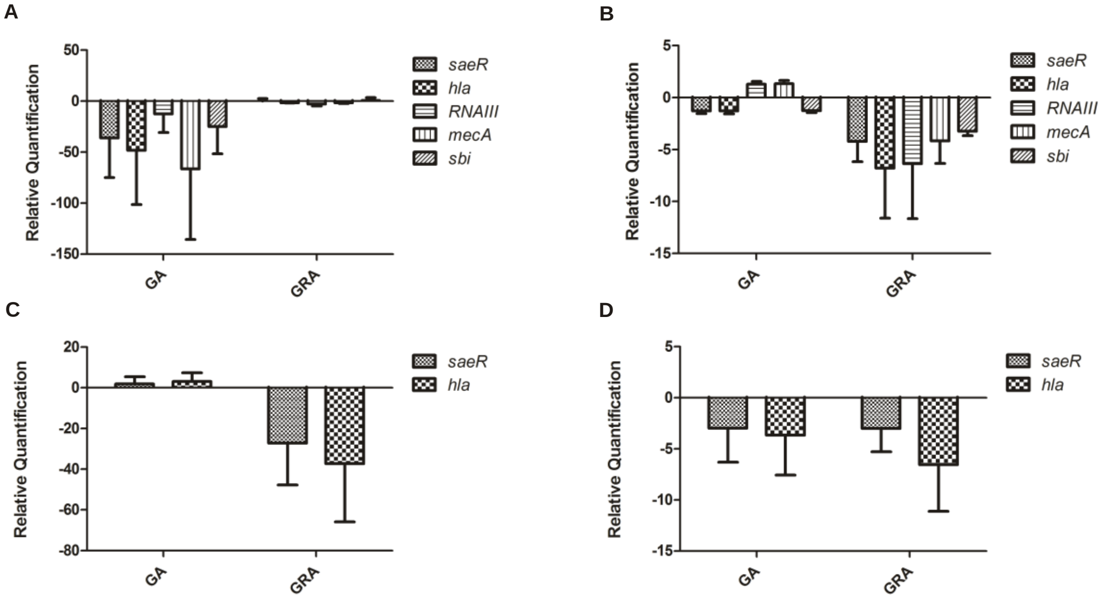

Fig. 5. GRA alters *S. aureus* virulence gene expression *in vivo*. Mice were inoculated subcutaneously with LAC (3×10^6) and treated with DMSO, GA, or GRA post-inoculation. At given time points, the site of inoculation was abscised and bacteria were recovered from the skin. Gene expression of five *S. aureus* virulence genes was investigated at (a) twelve and (b) twenty-four hours post-inoculation. Gene expression of two *S. aureus* virulence genes was investigated at (c) seventy-two hours and (d) ninety-six hours post-inoculation. Gene expression is normalized to *gyrB* expression and relative to *S. aureus* in media alone.

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CHAPTER 3

PROSPECTIVE STUDIES AND CONCLUSIONS

Determination of MIC

The development of new drug therapies against MRSA infections of all types is an important undertaking, but is expensive and time-consuming. Any new antimicrobial agent will have to undergo many costly tests to ensure MRSA does not become readily resistant, toxicity is non-existent or extremely low, efficacy is targeted to MRSA, and the agent must be effective against various infection types (2,5). We used agar dilution plates to determine the minimum inhibitory concentration (MIC) of GRA against MRSA (Fig. 1). MRSA was grown on TSA containing varied concentrations of GRA and GA. MRSA grew on plates containing 52.5 µg/ml and lower concentrations of GRA, but did not grow on plates containing higher concentrations of GRA. No concentration of GA was inhibitory to MRSA growth. Based on these results, we determined the MIC of GRA to be 60 µg/ml. Future studies, including serial passage of MRSA on TSA agar containing GRA at the MIC and determination of genetic variation in resistant colonies, will need to be completed to determine if GRA can induce resistance in MRSA.

Survival of MRSA in GRA-Treated Blood Fractions

The bactericidal effects of licorice root are well documented (6,9,13,14,18). We investigated whether sublethal doses of GRA would decrease the survival of MRSA in whole blood and serum. Bacteria were incubated in varied volumes of human whole blood or 20% human serum with a sublethal concentration of GRA or GA (52.5 µg/ml) for one hour. CFUs were enumerated and percent survival was calculated. There was no significant difference in the survival of MRSA in serum between the treatment groups (Fig. 2). In whole blood, the survival from all treatment groups was below 5%. The low survival rate is most likely due to severe clumping of bacteria and immune cells that was observed in the samples. Additional studies need to be completed which investigate the optimum conditions for this experiment. Blood and serum volume, incubation time, and a technique to disintegrate the blood clots and bacterial aggregates need to be explored further.

GRA Reduces Lethality of MRSA Infection

Our research has shown that topical treatment with GRA can reduce the severity of skin infections caused by MRSA. *S. aureus* skin and soft tissue infections, although they are becoming more prevalent and are difficult to treat, are not the only health concern arising from MRSA infections. MRSA can cause bacteremia, endocarditis, and sepsis, conditions which have high morbidity and mortality rates (10,16,17). These infections are becoming more difficult to treat due to an increase in antibiotic resistance (10,16,17). We investigated the ability of GRA to prevent morbidity caused by a mouse peritonitis model. The peritonitis model has been developed as a way to study the effects of antimicrobials against *S. aureus*, effectively utilizing a whole organism system with a functioning immune system and allowing pharmacokinetics to occur naturally (19). Mice received an intra-peritoneal injection of MRSA (~2×10^7 CFU) twelve hours after intra-peritoneal treatment with 250 µg GRA or GA suspended in PBS or PBS alone as a control. Mice received a second treatment one hour post-inoculation. All infected mice became sick (based on observations of mobility, food and water intake, weight loss, and respiratory distress) within twelve hours of inoculation. All mice recovered within two days, however, mice that received GRA treatment recovered more quickly than GA or PBS treated mice (data not shown). We also investigated the ability of GRA to prolong mouse survival after a lethal intra-venous injection of MRSA. Mice treated orally with GRA survived six to twelve hours longer than their GA or PBS treated counterparts (Fig. 3, data not significant). These preliminary findings are favorable to the development of GRA as a future antimicrobial, however future studies to investigate the effects of GRA on invasive MRSA infections will need to be refined to determine the ideal inoculation, time course of treatment, and physiologically relevant treatment doses.

Defining the Immune Response to GRA

A proper immune response is critical in host defense against pathogens. For defense against *S. aureus*, the neutrophil, or polymorphonuclear leukocyte (PMN), is extremely important. Individuals with neutrophil defects are extremely susceptible to infection by *S. aureus* (4,7,8). However, it has been shown that too robust of a neutrophil response can be detrimental to the host due to the release of cytotoxic factors from apoptotic PMNs (7,8). Thus, a measured immune response, with minimal pro-inflammatory responses, to MRSA infection may result in an ideal outcome, with rapid clearance of the pathogen and minimal damage to the host.

Published studies have shown GA and GRA to be anti-inflammatory. GA has been shown to inhibit the arachidonic cascade pathway, decrease IL-6 and IL-1 levels, reduce oxidative stress, activate the PI3K/Akt pathway, reduce reactive oxygen species generated by neutrophils, decrease IFN-β levels, reduce expression of pro-inflammatory genes for cytokines such as IL-1α, IL-1β and MIP-1α, block TNF-α release, and inhibit N_F-κB and MAPK pathways (1,11,15,20,21). GRA has been shown to inhibit inflammation through glucocorticoid receptor signaling, decrease IL-6 levels, and inhibit the classical complement pathway (11,12).

To investigate the effects of GA and GRA on the immune system, excluding infection, we gave mice an intra-peritoneal injection of GA or GRA suspended in PBS or PBS alone as a control. At specified time points after injection, the mice were sacrificed and peritoneal exudates were analyzed for cytokine presence, cellular influx, and neutrophil levels. The results from the cytokine assays were inconclusive due to the exudates being too dilute for analysis (data not shown). Exudates were stained with Diff-Quick and analyzed for cellular content. The exudates from mice receiving GRA treatment contained a much higher number of total cells than the exudates from mice receiving GA or PBS (Fig. 4). To further evaluate the contents of the exudates, we stained the cells with neutrophil markers Ly6G and CD11b and analyzed the population by flow cytometry. The exudates from GRA treated mice had significantly higher neutrophil content than exudates from mice receiving GA or PBS (Fig. 5). The influx of neutrophils seems to contradict previous findings on the anti-inflammatory effects of GRA. However, to our knowledge there are no published studies on the direct effect of GRA on neutrophil recruitment and chemotaxis. Also, the concentration of GRA used may result in different effects from previous studies. Future studies will further define the effect of GRA and GA on the immune system during normal and infected states.

Conclusions

Antimicrobial resistance continues to be one of the most pressing healthcare concerns. Resistance to current antibiotics is on the rise and there are few options currently being investigated (2,3). In this study we investigate the effects of a licorice root extract, 18-β-glycyrrhetinic acid, on the survival and virulence gene expression of methicillin-resistant *Staphylococcus aureus*. We demonstrated the dose-dependent bactericidal activity of GRA and also showed that GA has no direct effect on MRSA survival *in vitro*. Topical treatment with GRA significantly decreased the size of a skin abscess resulting from MRSA infection when compared to GA and control treated skin abscesses, however this reduction in size was not due to a reduction in bacterial burden. Further analysis of the infected tissue showed an alteration in the cytokine response to infection after treatment with GRA, implicating an effect of GRA on the immune system. Direct application with GRA resulted in a decrease in the expression of four key virulence genes in MRSA and an increase in the transcript of RNAIII, the effector of the Agr quorum-sensing system. GRA topical treatment of skin infections cause by MRSA also resulted in a down-regulation of five *S. aureus* virulence genes.

Determining if MRSA can develop resistance to GRA is an important step in evaluating the future use of GRA as an effective treatment against MRSA infections. We have determined the MIC of GRA, and future studies will need to be completed to investigate if, and how quickly, MRSA develops resistance to GRA. GRA has also shown promise as a treatment option against invasive MRSA infections, including peritonitis and bacteremia. Further investigation into the full effect of GRA against invasive infection is yet to be accomplished. The immune system plays a large role in the pathogenesis of MRSA, and we have shown that GRA is able to induce neutrophil-heavy cellular influx in the peritoneum. Further investigation into the effect of GRA on the immune system excluding infection, and during infection, will determine the mechanism behind this influx and whether the increase in immune cells is beneficial to clearance of infection or detrimental to the host. This study is a first step in determining if GRA has potential for future use as a therapeutic against MRSA skin and soft tissue and invasive infections, and provides a foundation for future work elucidating the full effect of GRA on both a pathogenic microorganism and the host immune system.

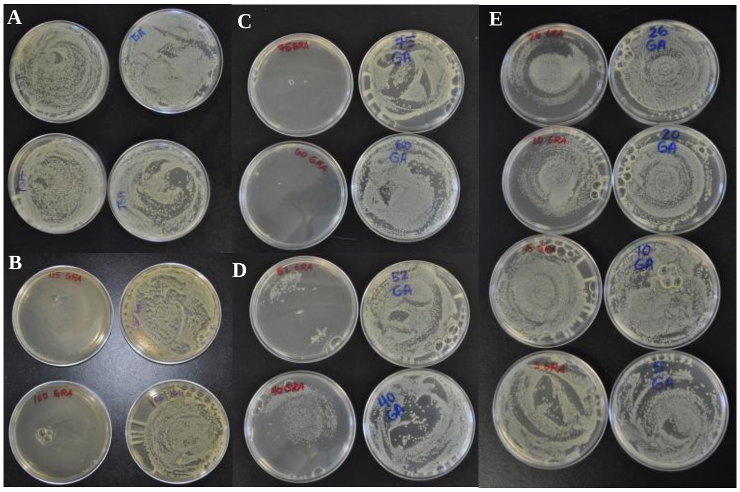

Fig. 1. Bactericidal effect of GRA on MRSA grown on agar. Approximately 10^4 CFUs LAC was plated on tryptic soy agar (TSA) and incubated overnight. TSA contained varying concentrations of GRA and GA to determine MIC. (A) Growth on TSA-only plates used for positive control. (B) No growth was seen on plates containing 125 µg/ml or 105 µg/ml GRA, but growth equal to the control was seen on TSA plates containing 125 or 105 µg/ml GA. (C) Only one or two colonies grew on TSA containing 75 µg/ml and 60 µg/ml GRA, respectively. Growth equal to the control was seen on TSA plates containing 75 or 60 µg/ml GA. (D) Approximately 70 CFUs grew on TSA containing 52.5 µg/ml GRA and an un-enumerable amount of colonies grew on TSA containing 40 µg/ml. Growth equal to the control was seen on TSA containing 52.5 or 40 µg/ml GA. (E) Un-enumerable colonies grew on TSA plates containing 26.25 and 20 µg/ml GRA, but the growth was not equal to that seen on the control TSA. Growth equal to the control was seen on TSA plates containing 10 or 5 µg/ml GRA, and TSA containing 26.25, 20, 10, or 5 µg/ml GA.

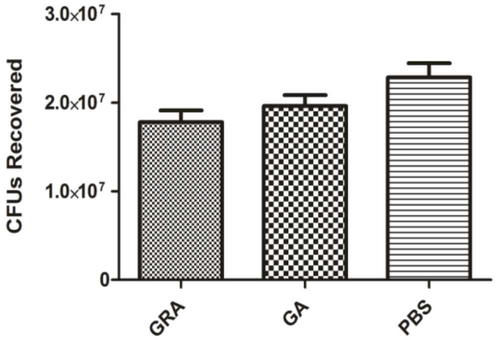

Fig. 2. Sub-lethal concentrations of GRA do not affect survival of LAC in serum. Approximately 2×10^7 CFUs were incubated in 20% human serum containing 52.5 µg/ml GRA or GA, or sterile PBS, for one hour. Survival of LAC in all three treatment groups was approximately 100%.

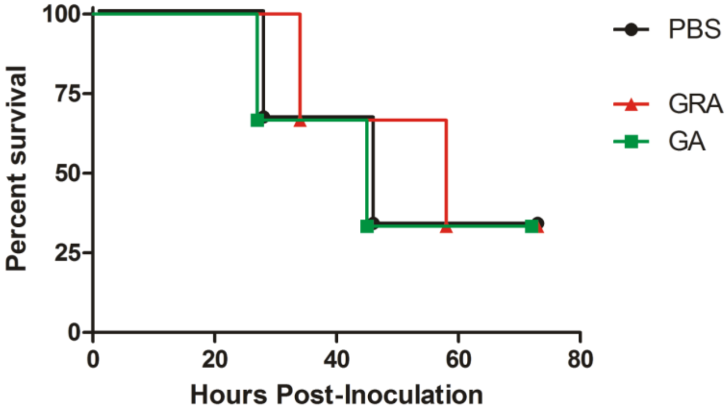

Fig. 3. Intra-peritoneal treatment with GRA prolongs mouse survival after infection with MRSA. Mice received a dose of approximately 2×10^7 LAC via intra-venous injection through the tail vein. Mice received a dose of GRA, GA, or PBS via oral gavage twelve hours prior to, and one and twenty-four hours after, inoculation. Mice were checked every three hours after inoculation and euthanized if moribund. Graph shows survival of mice over a seventy-two hour period.

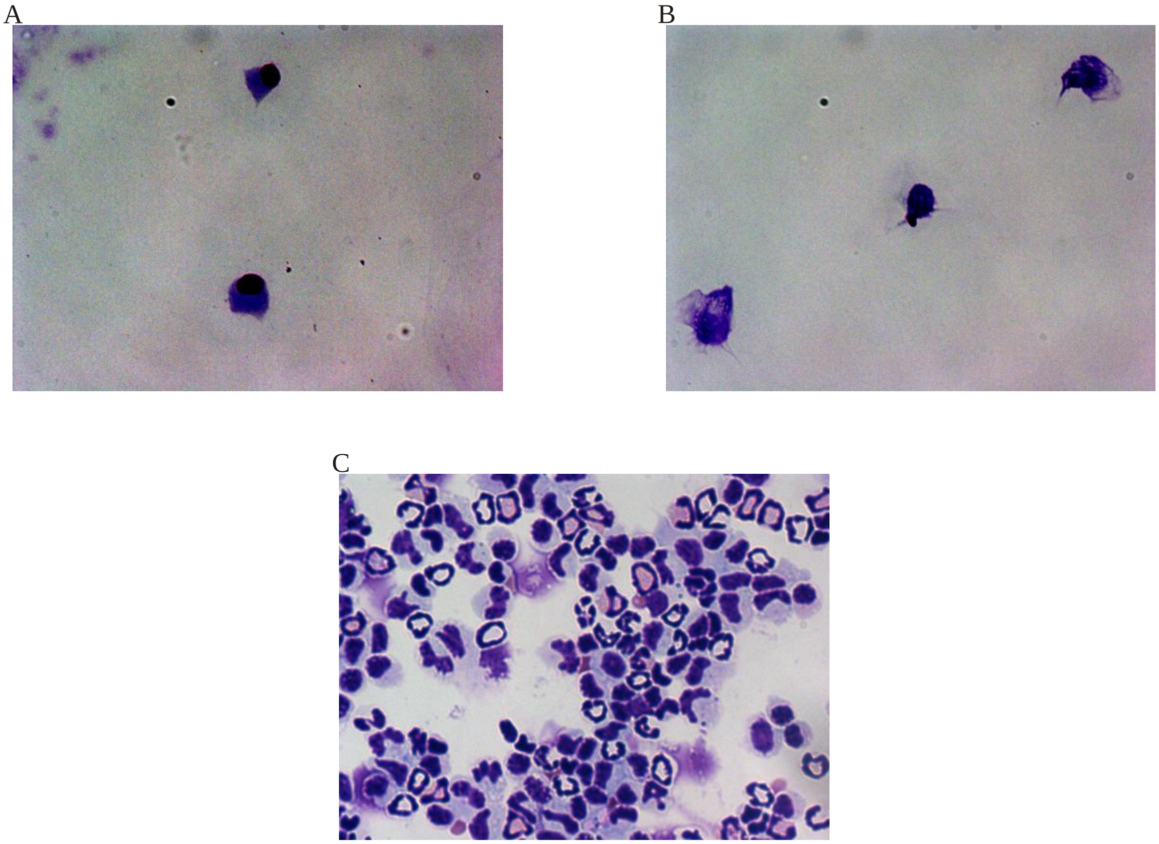

Fig. 4. Intra-peritoneal injection of GRA increased cellular influx into the peritoneal cavity. Mice received an intra-peritoneal dose of 600 µg GRA or GA suspended in 100 µl sterile PBS, or PBS alone. Twenty-four hours post-injection, the mice were sacrificed and peritoneal exudates were collected. The cellular exudates were stained using Diff-Quick and viewed under a microscope (40x) to determine cellular make-up of the exudates. Peritoneal fluid from mice injected with (A) PBS and (B) GA contained far fewer cells than peritoneal fluid from mice injected with (C) GRA. Photos are representative of three different mice with similar results.

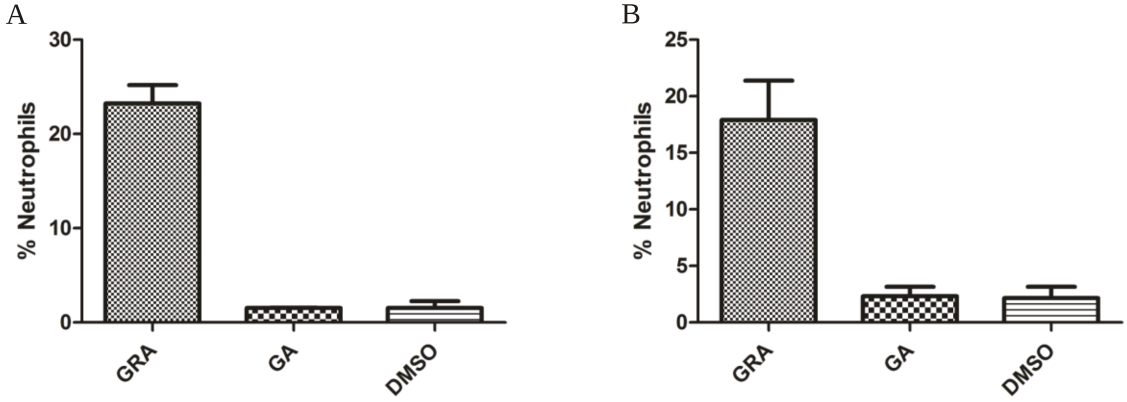

Fig. 5. Intra-peritoneal injection of GRA increases neutrophil influx into the peritoneal cavity. Mice were given an intra-peritoneal injection of 600 µg GRA or GA suspended in 100 µl PBS, or PBS alone. (A) Eight or (B) twenty-four hours later, the mice were sacrificed and peritoneal exudates were collected. The exudates were analyzed for neutrophil content via flow cytometry.

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