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Small Molecule Inhibitors Limit Endothelial Cell Invasion by *Staphylococcus aureus*

Diana Cordero^a, Christopher R. Fullenkamp^b, Rachel R. Pelly^b, Katie M. Reed^a, Lindy M. Caffo^a, Ashley N. Zahrt^a, Micaiah Newman^a, Sarah Komanapalli^a, Evan M. Niemeier^a, Derron L. Bishop^c, Heather A. Bruns^a, Mark K. Haynes^d, Larry A. Sklar^d, Robert E. Sammelson^b, and Susan A. McDowell^a

^aDepartment of Biology, Ball State University, Muncie, IN, USA, 47306

^bDepartment of Chemistry, Ball State University, Muncie, IN, USA, 47306

^cIndiana University School of Medicine - Muncie Campus, Muncie, IN, USA, 47306

^dCenter for Molecular Discovery, University of New Mexico, Albuquerque, NM, USA, 87131

Abstract

Staphylococcus aureus is a leading causative agent in sepsis, endocarditis, and pneumonia. An emerging concept is that prognosis worsens when the infecting *S. aureus* strain has the capacity to not only colonize tissue as an extracellular pathogen, but to invade host cells and establish intracellular bacterial populations. In previous work, we identified host CDC42 as a central regulator of endothelial cell invasion by *S. aureus*. In the current work, we report that ML 141, a first-in-class CDC42 inhibitor, decreases invasion and resultant pathogenesis in a dose-dependent and reversible manner. Inhibition was found to be due in part to decreased remodeling of actin that potentially drives endocytic uptake of bacteria/fibronectin/integrin complexes. ML 141 decreased binding to fibronectin at these complexes, thereby limiting a key pathogenic mechanism used by *S. aureus* to invade. Structural analogs of ML 141 were synthesized (designated as the RSM series) and a subset identified that inhibit invasion through non-cytotoxic and non-bactericidal mechanisms. Our results support the development of adjunctive therapeutics targeting host CDC42 for mitigating invasive infection at the level of the host.

Keywords

CDC42; fibronectin; ML 141; MRSA; MSSA; pyrazolines; sepsis

INTRODUCTION

S. aureus invasive infection is a multi-factorial process associated with life-threatening disease [1, 2]. Recent epidemiologic surveys identified methicillin sensitive *S. aureus*

Corresponding author: Susan A. McDowell, Ph.D., Cooper Science Complex, CL 171C, 2111 Riverside Ave., Ball State University, Muncie, IN 47306, Phone: +1-765-285-8846, FAX: +1-765-285-8804, samcdowell@bsu.edu.

CONFLICT OF INTEREST

For the remaining authors, none were declared.

(MSSA) strains in 50-60% of invasive *S. aureus* infections [1, 3]. Incidence of invasive infection due to methicillin resistant *S. aureus* (MRSA) appears to have plateaued near 50% and in some regions, to be declining [1, 2]. MSSA is reemerging as a leading causative agent in health-care-associated invasive infections [4] as MRSA is an emergent pathogen in community-onset invasive infection [2]. Improvements in preventative measures within healthcare settings are associated with recent declines in overall incidence, yet mortality associated with invasive infection by both MSSA and MRSA strains remains elevated [2, 5-7]. This indicates that although the infecting *S. aureus* strain may be susceptible to the current vanguard of antibiotic therapies, progression to life-threatening disease continues. Effective treatment strategies remain to be identified that mitigate the disease progression.

Historically, *S. aureus* had been described primarily as an extracellular pathogen and pathogenesis had been attributed largely to extracellular toxin production and colonization [8]. However, emerging characterization of invasive strains has begun to reveal multiple roles of host cell invasion in pathogenesis [9]. Host cell invasion is implicated as a potential mechanism for escape by *S. aureus* across blood vessels and metastasis into secondary infection sites that characteristically develop in survivors following sepsis [10]. The process of invasion is progressively damaging to endothelial cells [11] in part due to specialized toxin production initiated only after internalization [12]. Once internalized, intracellular populations elicit proinflammatory and procoagulant mediators, leading to further damage of host tissue [13]. Invasive *S. aureus* strains were found to initiate more extensive damage to endocardial tissue than non-invasive strains in a rodent model of infective endocarditis [13], and increased sepsis-associated mortality [14]. Intracellular *S. aureus* populations potentially evade extracellular antibiotics and immune cell surveillance, protected within the intracellular niche to reemerge in chronic, relapsing infection [8, 11, 15]. Although intracellular populations have been identified in clinical samples, questions remain regarding their viability and their contribution to pathogenesis [15]. Understanding the role of endothelial cell invasion in the multifaceted pathogenicity of *S. aureus* has the potential to improve outcomes and to address morbidity and mortality that characterize invasive infection by this pathogen.

S. aureus invades host cells by exploiting the $\alpha 5 \beta 1$ integrin receptor and its ligand fibronectin [9]. Fibronectin-binding proteins on the surface of invasive *S. aureus* strains bind host fibronectin. When bacterial-bound fibronectin attaches to $\alpha 5 \beta 1$, internalization is stimulated, taking the bacterial cargo into the host cell. Concomitantly, actin stress fibers disassemble [16]. Actin stress fibers are contractile bundles of actin filaments and this remodeling potentially provides traction necessary for internalization of the fibronectin/bacteria/integrin complexes [17].

Previously, we found that cholesterol-lowering simvastatin decreased endothelial cell invasion by *S. aureus* [16] and improved survival in a murine model of pneumonia [18]. The underlying pharmacology is due in part to decreased formation of isoprenoid intermediates within the cholesterol biosynthesis pathway. Isoprenoid intermediates serve as membrane anchors for proteins possessing the CaaX domain [19]. Through covalent binding of hydrophobic isoprenoid groups to the cysteine residue within the CaaX domain, prenylated proteins acquire membrane localization, engage in protein-protein interactions, and access

downstream effector molecules. We examined Rac, Rho B, and CDC42, CaaX-domain containing proteins that regulate receptor-mediated endocytosis. We found that simvastatin led to a loss in membrane localization of each [16]. Earlier work had indicated that CDC42 can function upstream of Rac and Rho B in the regulation of actin remodeling [20]. We used site-directed mutagenesis to substitute the cysteine residue within the CaaX-domain of CDC42 with valine and found that loss of this singular GTPase's prenylation site decreased invasion by 90% [16]. The finding suggested that CDC42 serves as a central regulatory protein used by *S. aureus* to invade.

In the current work, we examined potential regulatory roles of CDC42 during the invasive process and assessed whether small molecule inhibition of host CDC42 would mitigate pathogenesis. For these studies, we used ML 141, a first-in-class, reversible, allosteric inhibitor that induces dissociation of guanine nucleotides (GDP and GTP) from the active site of CDC42 [21]. Predictive models suggest ML 141 would be aromatized readily *in vivo* and that low solubility may limit bioavailability. Structural analogs of ML 141 were synthesized (designated as the RSM series) and the pyrazolines screened for their ability to limit invasive infection through non-cytotoxic and nonbactericidal mechanisms.

MATERIALS AND METHODS

Endothelial cell culture and compound treatment

Human umbilical vein endothelial cells (HUVEC, EMD Millipore, Billerica, MA) were cultured in EndoGRO-LS media (EMD Millipore) and maintained at 5% CO₂, 37°C, in 75 cm² vented cap flasks (Thermo-Fisher, Pittsburgh, PA). For invasion assays, HUVEC were plated at 1×10⁵ cells/ml in 35 mm culture dishes (Thermo-Fisher) or at 1×10⁴ cells/ml in 96-well plates (Thermo-Fisher) coated with Attachment Factor (Life Technologies, Carlsbad, CA). The next day, HUVEC were pretreated in medium containing the vehicle control, ML 141, or RSM structural analog. ML 141 and RSM structural analogs were suspended in dimethyl sulfoxide (DMSO, Thermo Fisher Scientific) or in polyethylene glycol (PEG, Sigma-Aldrich, St. Louis, MO) at a concentration of 5 mmol/L. The 5 mmol/L solution was diluted to 1 mmol/L in the solvent and then diluted to the final concentration for each experiment in EndoGRO-LS media. For vehicle control treatment, the same volume of DMSO or of PEG as that of ML 141 or of the RSM structural analog was added to medium. In most experiments, the invasion assay was performed 18-20 h later. Because *in vitro* data indicate that ML 141 is a reversible inhibitor [22], bacteria were added directly to media containing vehicle control, ML 141, or the RSM structural analog.

Invasion assay

Two days prior to the assay, 5 ml tryptic soy broth cultures (TSB, Sigma-Aldrich) were inoculated with 10 µl ATCC 29213, a MSSA strain (American Type Culture Collection, Manassas, VA), with 10 µl MRSA252 NRS71, a hospital strain, or with 10 µl NRS123, a community-acquired MRSA strain. MRSA strains were provided kindly by Dr. Kathleen Dannelly, Indiana State University. Bacterial cultures were incubated overnight (225 rpm, 37°C), and subcultured the next day. On the following day, bacteria were pelleted (10000×g, 37°C, 3 min), washed in saline, pelleted as above, and resuspended in saline. For the MSSA

strain and for MRSA252 NRS71, the resuspended pellet was incubated with rabbit anti-mouse IgG Alexa Fluor 488 to fluorescently label bacteria (Life Technologies, final concentration 8 µg/ml, RT, 20 min). Protein A, a *S. aureus* cell surface protein, binds IgG thereby labeling the bacteria. Labeled bacteria were washed twice as above and resuspended to 3×10^8 colony forming units (CFU)/ml in saline. HUVEC were incubated with fluorescently-labeled bacteria (1 h, MOI 1440, 5% CO₂, 37°C), extracellular bacteria removed by extensive washes with phosphate buffered saline (PBS, Life Technologies) and incubation with 20 µg/ml lysostaphin/50 µg/ml gentamicin (Sigma Aldrich; 45 min, 5% CO₂, 37°C), and washed with PBS as previously described [16]. To detect fluorescent HUVEC (indicative of the infected population), HUVEC were lifted from culture dishes using cell scrapers, pelleted, fixed [1% bovine serum albumen (BSA, Thermo Fisher Scientific)/0.74% formaldehyde/PBS], and analyzed using an Accuri C6 flow cytometer (BD, Franklin Lakes, NJ) as described below. To assess invasion by the MRSA strain NRS123, HUVEC were incubated with non-fluorescently labeled bacteria at the same MOI (1 h, 5% CO₂, 37°C), extracellular bacteria removed as above, and intracellular bacteria released using 1% saponin (Sigma-Aldrich)/PBS (20 min, 5% CO₂, 37°C). Serial dilutions of bacteria-containing saponin were plated on tryptic soy agar (TSA, Sigma-Aldrich, 16 h, 37°C), and CFU/ml determined. For assessing intracellular bacterial populations 48 h post infection, HUVEC were incubated with non-fluorescently labeled MSSA (MOI 50, 1 h, 5% CO₂, 37°C), extracellular bacteria removed as above, and incubation continued in antibiotic-containing media. At 48 h post-infection, HUVEC were permeabilized with saponin as above, serial dilutions incubated on TSA (16 h, 37°C), and CFU/ml quantified.

CDC42 activation assay

HUVEC were serum starved in base media containing DMSO or ML 141 (18-20 h). *S. aureus* cultures were washed as described above, incubated with FBS (15 min, RT) as the source of fibronectin, washed extensively, resuspended in saline, and incubated with HUVEC (MOI 1440, 1 h, 5% CO₂, 37°C). Total protein concentration of lysates was determined, samples diluted to 0.125 mg/ml in lysis buffer containing protease inhibitors, and GTP-bound CDC42 detected using the G-LISA Cdc42 Activation Assay Biochem Kit (Cytoskeleton, Denver, CO). Of note, more concentrated lysates (0.25 mg/ml) appeared to saturate the assay.

Transmission electron microscopy

HUVEC were plated onto glass coverslips at 3×10^5 /ml, pretreated and infected (MOI 30, 2 h, 5% CO₂, 37°C). Extracellular bacteria were removed as above and HUVEC incubated for an additional 24 h in media containing antibiotics. HUVEC were immersion fixed with 2.5% glutaraldehyde/2.0% paraformaldehyde/2 mmol/L calcium/1 mmol/L magnesium (15 min, twice; Electron Microscopy Sciences, Hatfield, PA), washed extensively with PBS, post-fixed with 1.0% osmium tetroxide/1.5% potassium ferrocyanide in 0.1 mol/L sodium cacodylate buffer, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in EMBed812 (Electron Microscopy Sciences). Serial ultrathin sections were cut, collected onto Pioloform slot grids, and counterstained with aqueous uranyl acetate and Reynold's lead citrate (30 min each). Electron micrographs were obtained at 120KV using a JEOL JEM-1400 equipped with a Gatan Ultrascan 1000XP camera.

Immunofluorescence

For confocal imaging, HUVEC were plated at 3×10^5 /ml (for detection of actin) or at 3×10^4 (for detection of vinculin) on 35 mm glass-bottom dishes (MatTek, Ashland, MA) coated with Attachment Factor. HUVEC were pretreated with vehicle control or with ML 141. For analysis of actin stress fiber disassembly, HUVEC were infected (MOI 1200, 1 h, 5% CO₂, 37°C), washed with 1X PBS, fixed [4% paraformaldehyde (Electron Microscopy Sciences)/PBS, 30 min], permeabilized, blocked (0.1% Triton/1% BSA, 30 min), and incubated with Alexa Fluor 488 phalloidin (1:40; Life Technologies). For detection of adhesion complexes, HUVEC were fixed, permeabilized, and blocked as above, and incubated with anti-vinculin (Sigma-Aldrich, 1:25) followed with rabbit anti-mouse Alexa Fluor 488 (Life Technologies, 1:250). Actin stress fibers were detected using a Zeiss Axiovert200 microscope equipped with a plan-apochromat 40X, 1.2 NA water immersion lens with correction collar. Vinculin was detected using a Zeiss Axioskop 2 equipped with a 63X, water immersion lens. Confocal images were acquired using a LSM 5 Pascal scan head. Z-sectioning and frame size were set to Nyquist sampling and images generated from maximum pixel projections of Z-stacks.

Flow cytometry

To assess bacterial invasion, HUVEC, incubated with fluorescently-labeled bacteria and processed as described above, were analyzed for fluorescence intensity. HUVEC were identified through FSC-A versus SSC-A gating. Cells demonstrating size and granularity indicative of HUVEC were gated (1500 cells). Mean fluorescence intensity (MFI) of Alexa Fluor 488 from 1500 gated cells/sample was determined using CFlow Plus software (BD Accuri).

To examine $\beta 1$ surface expression, recycling of the integrin was terminated (4°C, 15 min) and HUVEC lifted from plates using cell scrapers. After washing in FACS buffer (2% BSA/0.1% sodium azide/PBS), HUVEC were incubated with PE-conjugated anti- $\beta 1$ (BD; 30 min, on ice), washed, fixed in FACS buffer containing 4% paraformaldehyde, and collected using the Accuri C6 flow cytometer. HUVEC were identified by FSC-A/SSC-A gating. MFI of PE from 600 gated cells/sample was analyzed using FlowJo vX software (TreeStar, Ashland, OR).

Fibronectin adhesion assay

HUVEC were plated and pretreated as described for the invasion assay. 96-well, non-tissue culture treated plates (Sarstedt, Newton, NC) were coated with human fibronectin (Sigma-Aldrich, 20 μ g/ml; 2 h; 37°C), washed with PBS, and blocked with BSA (2%, 30 min, RT). HUVEC were lifted from the 35 mm dishes using cell scrapers, 100 μ l counted for normalization using the Accuri C6, and 200 μ l transferred to the 96-well plate (2 h, 5% CO₂, 37°C). After extensive washes in PBS, adherent HUVEC were removed from the plate using trypsin (Life Technologies), washed in FACS buffer, fixed, 100 μ l counted, and counts normalized.

Cytotoxicity assay

HUVEC were plated and treated as described for the invasion assay. Following pretreatment, HUVEC were lifted from plates using cell scrapers, washed extensively in FACS buffer, incubated briefly with PI (Sigma-Aldrich) at 0.5 mg/ml, and fluorescence detected immediately using the Accuri C6 flow cytometer.

Bactericidal activity assay

1.2×10^8 CFU/ml were incubated with compound or with vehicle control in EndoGRO-LS media (1 h, 5% CO₂, 37°C). Serial dilutions were plated onto TSA, incubated (18-20 h, 37°C), colonies enumerated, and CFU/ml determined.

ML 141 and RSM structural analog synthesis

4-[3-(4-methoxyphenyl)-5-phenyl-3,4-dihydropyrazol-2-yl]benzenesulfonamide (ML 141) was generously provided by Angela Walldinger-Ness of Center for Molecular Discovery, University of New Mexico, Albuquerque, NM, by Dr. Jennifer Golden of the University of Kansas Specialized Chemistry Center, or was prepared following standard synthetic procedures [23].

General procedure for pyrazolines (RSM structural analogs) [23]

Equimolar equivalents of the corresponding chalcone (see below) and p-sulfamylphenylhydrazine hydrochloride [24] were dissolved in 95% ethanol (25 mL/mmol) and a catalytic amount of sodium acetate was added. The mixture was then refluxed for 24 h. The reaction mixture was concentrated to about 1/3 of the volume via simple distillation. The reaction was then cooled to room temperature and the product was isolated via vacuum filtration. When necessary, the product was recrystallized with ethanol or purified by flash chromatography on silica gel.

General procedure for chalcone synthesis

The corresponding benzaldehyde (1.0 equiv.) and the corresponding acetophenone (1.0 equiv.) derivatives were dissolved in 95% ethanol (1.5 mL/mmol) and cooled to 0°C in an ice bath [25]. A solution of 40% sodium hydroxide (up to 0.5 mL/mmol) was then slowly added drop wise over 1 h or until solid began to precipitate. The mixture was left to stir at 0°C for another 30 min and the product was isolated via vacuum filtration. The crude solid was washed with cold 95% ethanol and was recrystallized using 95% ethanol. Chalcone used for the preparation of RSM 26 was prepared following literature procedure for the cuprate addition to a conjugated ynone [26].

General procedure for alkylation of phenols [27]

4-Hydroxyacetophenone or 4-hydroxybenzaldehyde (1.00 equiv.) was added to a solution of 2-chloroethyl methyl ether (1.20 equiv.) and potassium carbonate (1.20 equiv.) in DMF (1.5 mL/mmol). The reaction mixture was heated to 60°C and left to stir for 16 h. The mixture was cooled, water (30 mL) added and extracted with ethyl acetate (3×30 mL). The combined organic layers were then washed with 1M NaOH (1×30 mL) water (3×30 mL) and brine

(1×30 mL) and dried over magnesium sulfate. The solvent was removed *in vacuo* and crude product used without further purification.

Statistical analysis

Differences between groups were considered statistically significant at $P < 0.05$. When comparing the mean of two groups, the P value was computed by Student's t -test for parametric data and by Mann-Whitney Rank Sum test for nonparametric data. When comparing the mean from three or more groups, the P value was computed using one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis. When comparing presence/absence of actin stress fibers or of vinculin-containing adhesion complexes, P values were computed by χ^2 test of association (Sigma Stat, Systat, Point Richmond, CA).

RESULTS

ML 141 limits host cell invasion by *S. aureus*

HUVEC were pretreated with ML 141 (10 $\mu\text{mol/L}$, 18-20 h) or with vehicle control and incubated (1 h) with Alexa Fluor 488-labeled ATCC 29213, an invasive MSSA strain. Extracellular bacteria were removed using antimicrobials with limited mammalian cell permeability. Infected HUVEC (identified by 488 fluorescence) were detected by flow cytometry. Mean fluorescence intensity was lower in HUVEC pretreated with ML 141 compared to vehicle control ($P < 0.05$ by Student's t -test, Fig. 1, Panel A). ML 141 inhibited invasion by MRSA252 NRS71, a hospital isolate, and by NRS123, a MRSA isolate of community origin ($P < 0.05$ by Student's t -test, Fig. 1, Panel B). The invasive MSSA strain ATCC 29213 was used for all subsequent analyses.

ML 141 inhibited invasion in a dose dependent manner ($\text{IC}_{50} = 5.4 \mu\text{mol/L}$; $P < 0.05$ by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis, Fig. 1, Panel C). To examine whether inhibition was reversible, HUVEC were pretreated with ML 141 (10 $\mu\text{mol/L}$) or with vehicle control (1 h). Following pretreatment, media containing ML 141 was replenished with vehicle control-containing media for 40 min prior to invasion. Mean fluorescence intensity returned to baseline in these washout samples ($P < 0.05$ by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis, Fig. 1, Panel D).

To determine whether inhibition led to decreased intracellular bacterial populations over time, HUVEC were pretreated with ML 141 (10 $\mu\text{mol/L}$) or with vehicle control (18-20 h), incubated with *S. aureus* (1 h), washed, and incubated in antimicrobial-containing media (48 h). The number of viable bacteria recovered at 48 h was 85% lower in ML 141 treated cells compared to vehicle control (data not shown, $P < 0.05$ by Student's t -test).

ML 141 inhibits CDC42 activation during *S. aureus* invasion

CDC42-GTP levels increased in HUVEC in response to invasion (1 h), indicative of CDC42 activation ($P < 0.05$ by Student's t -test, Fig. 2, Panel A). Following invasion (1 h), CDC42-GTP levels were not different in HUVEC pretreated (18-20 h) with ML 141 (10 $\mu\text{mol/L}$) compared to vehicle control ($P > 0.05$ by Student's t -test, Panel B).

ML 141 limits damage to host cells during invasive infection

To examine whether inhibition of invasion and sustained suppression of intracellular bacterial populations limits damage to host cells, HUVEC were pretreated (18-20 h) with ML 141 (10 $\mu\text{mol/L}$) or with vehicle control, infected (2 h), and extracellular bacteria removed. HUVEC were examined at the ultrastructural level 24 h post-invasion. Following invasion, organelles that remained intact within ML 141 treated cells were not detectable within vehicle control treated cells (Fig. 3). At the host cell membrane, filopodia that form in response to bacteria were not detected in ML 141 treated cells, yet were readily observed in the vehicle control. Bacteria within ML 141 treated cells were located within membranous boundaries. In vehicle control treated cells, bacteria were cytosolic and appeared to be undergoing cellular division.

ML 141 limits actin stress fiber disassembly during *S. aureus* invasion

At day 2 of plating, HUVEC displayed extensive stress fiber formations (Fig. 4). Stress fibers were no longer detectable in 75% of infected (1 h) vehicle control treated cells, but remained intact in infected ML 141 treated cells ($P < 0.05$ by χ^2 test of association, 10 $\mu\text{mol/L}$, 18-20 h).

ML 141 decreases adhesion complex formation and function

ML 141 treatment decreased adhesion complexes from 64% in vehicle control treated cells to 37% in ML 141 treated cells ($P < 0.05$ by χ^2 test of association, 10 $\mu\text{mol/L}$, 18-20 h, Fig. 5, Panel A). No difference in expression of $\beta 1$ was detected between treatment groups (Mann-Whitney Rank Sum test, $P = 0.10$, Fig. 5, Panel B). $\beta 3$ expression was not detected in HUVEC (data not shown). ML 141 treatment (10 $\mu\text{mol/L}$, 18-20 h) decreased HUVEC adhesion to fibronectin ($24 \pm 4\%$ to $14 \pm 2\%$, $P < 0.05$ by Student's t -test, Fig. 5, Panel C).

Structural analogs of ML 141 limit host cell invasion

Structural analogs were synthesized (designated as the RSM series, Table 1 in Materials and Methods). Mean fluorescence intensity was measured from HUVEC pretreated with RSM structural analogs or vehicle control and incubated with fluorescently labeled *S. aureus* as in Fig. 1. Mean fluorescence intensity was lower in response to a subset of RSM structural analogs (Table 2).

To determine whether the decrease in fluorescence was due to cytotoxic or to bactericidal activity rather than due to inhibition of invasion, cytotoxicity and bactericidal assays were performed. Cytotoxicity was detected in HUVEC incubated (1 h) with RSM 04 or with RSM 26 at 10 $\mu\text{mol/L}$ (Table 3). Cytotoxicity was not detected in response to ML 141 or any other RSM structural analog at this concentration. RSM 26 was bactericidal at 10 $\mu\text{mol/L}$ (Table 3). Bactericidal activity was not detected following incubation of MSSA with ML 141 or with any other RSM structural analog at 10 $\mu\text{mol/L}$ (Table 3). Bactericidal activity was not detected when either MRSA strain was incubated with ML 141. For MRSA252 NRS71, $1.9 \times 10^{10} \pm 0.2$ CFU/ml were recovered following incubation of 1.2×10^8 CFU/ml (1 h) with ML 141 (10 $\mu\text{mol/L}$) or with vehicle control. For NRS123, $5.6 \times 10^{10} \pm 1.5$ CFU/ml were

recovered following incubation of 1.2×10^8 CFU/ml with ML 141 vs. $4.5 \times 10^{10} \pm 0.6$ CFU/ml recovered following incubation with vehicle control ($P > 0.05$ by Student's *t*-test).

DISCUSSION

In this study, we examined whether ML 141, a highly selective CDC42 inhibitor, disrupts cellular processes used by *S. aureus* for invasion. Our results indicate ML 141 decreased invasion by MSSA and MRSA strains (Fig. 1) through mechanisms that are neither cytotoxic nor bactericidal (Table 3). ML 141 diminished CDC42 activation (Fig. 2) and mitigated host cell damage (Fig. 3). Inhibition appears to be mediated in part by impeding the remodeling of actin that potentially drives endocytic uptake of bacteria/fibronectin/integrin complexes (Fig. 4). ML 141 treatment decreased formation of integrin complexes at the host cell surface and decreased adhesion of the host cell to fibronectin (Fig. 5). Although other invasive mechanisms have been identified, fibronectin-binding appears to be the primary route used by pathogenic *S. aureus* strains for establishing intracellular, persisting infection [28]. By decreasing the abundance of adhesion complexes and associated disruption in adhesion to fibronectin, our findings indicate that targeted inhibition of CDC42 limits the principal mechanism used by *S. aureus* for invasion.

It remains to be determined whether small molecule inhibition of CDC42 improves rather than impedes bacterial clearance *in vivo*. Usefulness of ML 141 for addressing this question *in vivo* may be limited due to predicted low solubility and the propensity to aromatize. We therefore synthesized the RSM series, structural analogs of ML 141. A subset of the RSM series inhibited invasion through non-cytotoxic and non-bactericidal mechanisms (Tables 2-3). Once the subset is characterized for specificity toward CDC42, solubility, and predicted routes of metabolism, these structures will provide a framework for the development of compounds with *in vivo* efficacy.

Decreased invasion in response to ML 141 appears to be mediated through inhibition of CDC42 and through effects on host cellular processes under the regulation of CDC42. In support of this concept, increases in CDC42-GTP in HUVEC during invasion were diminished in ML 141 treated cells (Fig. 2). ML 141 is a reversible non-competitive inhibitor selective for CDC42 [22]. Our finding that CDC42-GTP levels remain at baseline in infected cells treated with ML 141 would be consistent with inhibition at this binding site. Inhibition of GTP-binding by ML 141 potentially disrupts interactions with downstream effector proteins that mediate the host cell response to *S. aureus* invasion. During host cell invasion by *S. aureus*, actin stress fibers disassemble [16]. The change in actin architecture is consistent with CDC42 activation and of the downstream effector WASP [29]. In response to ML 141, actin stress fibers remained intact in response to *S. aureus*, suggesting that ML 141 inhibition of CDC42 disrupts the remodeling of actin that potentially drives bacterial internalization. Further support for the concept that ML 141 is working through CDC42 to limit invasion is that the observed decrease in formation of adhesion complexes and the decrease in adhesion of the host cell to fibronectin is consistent with earlier findings in CDC42^{-/-} mouse embryonic fibroblasts [30, 31]. Taken together, our findings suggest that ML 141 inhibits invasion by impeding host cell functions under the regulation of this small GTPase.

ML 141 treatment appears to diminish adhesion complex formation through a mechanism that disrupts assemblage of these complexes rather than through a mechanism that decreases expression of the central organizing integrin as $\beta 1$ expression remained unchanged. Our finding is consistent with earlier work in CDC42^{-/-} fibroblasts where $\beta 1$ expression was comparable to wild type controls [30]. Thus, inhibition of CDC42 can impede assemblage of components independently of effects on $\beta 1$ expression. Although $\beta 1$ expression remains unchanged in CDC42^{-/-} fibroblasts and in response to ML 141, expression decreased following siRNA knockdown of CDC42 [32]. Differing effects on $\beta 1$ expression may reflect the different strategies used for decreasing functional CDC42 (targeted genetic knock-out, pharmacologic, or siRNA). However, regardless of the strategy used, adhesion to fibronectin consistently decreases when CDC42 is functionally inactivated (Fig. 5) [30-32]. Our results indicate that in the absence of functional CDC42, adhesion to fibronectin is diminished and that the decrease in adhesion can be through mechanisms independent of $\beta 1$ expression levels.

Decreased host cell adhesion to fibronectin in response to ML 141 may have implications that extend beyond *S. aureus* infection. In addition to *S. aureus*, the intracellular pathogens *Coxiella burnetii*, *Chlamydia*, *Legionella*, and *Bartonella*, potentially use fibronectin-binding to invade endocardial tissue during infective endocarditis [13]. Fibronectin-binding facilitates invasive infection by *Mycobacterium leprae*, a causative agent of leprosy [33], *Neisseria gonorrhoeae*, the sole causative agent of gonorrhea [34], and by *Streptococcus pyogenes*, a re-emergent pathogen characterized by aggressive invasive infection in strains positive for fibronectin-binding proteins [35]. Our results raise the possibility that small molecule inhibition of CDC42 would limit infection of clinically important pathogens reliant on fibronectin-binding mechanisms for invasion.

CONCLUSION

Invasive *S. aureus* strains initiate progressive tissue damage and potentially establish intracellular bacterial populations that reemerge as chronic, relapsing infection [8, 11, 15]. The current work revealed ML 141 decreased host cell invasion and mitigated host cell damage, raising the possibility that inhibition of CDC42 would increase extracellular antibiotic efficacy and limit establishment of persisting intracellular populations, reducing infection-related morbidities and mortality. These findings support further development of small molecule inhibitors with specificity toward host CDC42 as adjunctive therapeutics in the treatment of invasive infection by *S. aureus* and potentially by other clinically important pathogens that rely on fibronectin for invasive infection.

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

ML 141	4-[3-(4-methoxyphenyl)-5-phenyl-3,4-dihydropyrazol-2-yl]benzenesulfonamide
HUVEC	human umbilical vein endothelial cells
MOI	multiplicity of infection
CFU	colony forming unit
DMSO	dimethyl sulfoxide
PEG	polyethylene glycol
TSB	tryptic soy broth
TSA	tryptic soy agar
siRNA	short interfering RNA

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Figure 1.

ML 141 decreases host cell invasion. **A.** Human umbilical vein endothelial cells (HUVEC) were pretreated (18-20 h) with vehicle control polyethylene glycol (PEG) or with ML 141 (10 μ mol/L) suspended in PEG. Following infection by Alexa Fluor 488-labeled ATCC 29213, a methicillin susceptible *S. aureus* strain (1 h), extracellular bacteria were removed using antimicrobials that have limited mammalian cell permeability. Mean fluorescence intensity values (x-axis) are represented by histogram overlay with the y-axis indicating the number of HUVEC identified by FSC-A versus SSC-A gating. Data also are represented as percent control $\pm$ SEM. (*less than control, $P < 0.05$ by Student's *t*-test; $n = 3$ /treatment). **B.** ML 141 decreases host cell invasion by methicillin resistant *S. aureus* (MRSA) strains. HUVEC were pretreated as above, infected with MRSA252 NRS71, a hospital strain, or with NRS123, a community-acquired MRSA strain, and invasion assessed. **C.** Decrease in invasion is dose dependent. HUVEC were pretreated with increasing concentrations of ML 141, infected (1 h), and invasion assessed. Data presented as dose-response curve. $IC_{50} = 5.4$ μ mol/L (*less than control, $\dagger$ less than 2.5 μ mol/L; $\ddagger$ less than 5 μ mol/L, $P < 0.05$ by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis; $n = 4$ /treatment). **D.** Inhibition of invasion is reversible. HUVEC were pretreated with PEG or with ML 141 (1 h, 10 μ mol/L). Following pretreatment, ML 141-containing media was removed from the washout samples and replaced with PEG-containing media for 45 min prior to infection. Invasion was measured as above (*less than control, $P < 0.05$ by Student's *t*-test; $n = 5$ /treatment).

Figure 2.

Invasion by *Staphylococcus aureus* stimulates host CDC42 activity. **A.** Human umbilical vein endothelial cells (HUVEC) were serum starved (18-20 h) and incubated with fibronectin-coated *S. aureus* (1 h). Following infection, CDC42 activation was measured in cell lysates. **B.** ML 141 inhibits CDC42 during *S. aureus* invasion. HUVEC were pretreated with vehicle control dimethyl sulfoxide (DMSO) or with ML 141 (10 μ mol/L) in serum-free media (18-20 h), and incubated with fibronectin-coated *S. aureus* (1 h). CDC42 activity was measured in cell lysates. Data are presented as fold control $\pm$ SEM (* P < 0.05 by Student's t -test; n = 4/treatment).

Figure 3.

ML 141 limits damage to host cells. Human umbilical vein endothelial cells (HUVEC) were incubated with vehicle control polyethylene glycol (PEG) or with ML 141 (10 $\mu\text{mol/L}$) 18-20 h prior to infection with *Staphylococcus aureus* (2 h). Following infection, extracellular bacteria were removed by incubating with lysostaphin and gentamicin (45 min), antimicrobials with limited mammalian membrane permeability. Antimicrobial-containing media was replenished and incubation extended an additional 24 h. HUVEC were fixed, stained, and examined by transmission electron microscopy. White arrow indicates mitochondria. Black arrow indicates filopodia. White arrowhead indicates membranous structure surrounding bacteria. Black arrowhead indicates dividing bacteria. Scale bar is 5 μm .

Figure 4.

ML 141 decreases actin stress fiber depolymerization during infection. Human umbilical vein endothelial cells (HUVEC) were incubated with vehicle control polyethylene glycol (PEG) or with ML 141 (10 $\mu\text{mol/L}$) 18-20 h prior to infection with *Staphylococcus aureus* (1 h). Actin was detected using Alexa Fluor 488 phalloidin. Arrows indicate intact actin stress fibers. Arrowhead indicates absence of actin stress fibers. Data are presented as the percentage of HUVEC with no detectable actin stress fibers. 100-200 cells/treatment were evaluated from randomly selected fields. Scale bar is 50 μm ($P < 0.05$ by χ^2 test of association).

Figure 5.

ML 141 limits the formation of adhesion complexes and adherence to fibronectin. Human umbilical vein endothelial cells (HUVEC) were incubated (18-20 h) with vehicle control dimethyl sulfoxide (DMSO) or with ML 141 (10 $\mu\text{mol/L}$) and adhesion complex formation and function assessed. **A.** Vinculin-containing adhesion complexes diminish with ML 141 treatment. Pretreated HUVEC were fixed, permeabilized, blocked, and stained with anti-vinculin followed by anti-mouse Alexa Fluor 488. Arrows indicate vinculin-containing complexes. Data are presented as the % of HUVEC where vinculin-containing adhesion complexes were detected. 100-200 cells/treatment were evaluated from randomly selected fields ($P < 0.05$ by χ^2 test of association). Scale bar 50 μm . **B.** Expression of the $\beta 1$ integrin subunit remains unchanged. Pretreated HUVEC were stained with PE conjugated anti- $\beta 1$ and examined using flow cytometry. Data are represented as percent control $\pm$ SEM (no difference between groups was detected, Mann-Whitney Rank Sum test, $P = 0.10$) and by histogram overlay (dark gray represents DMSO control, light gray represents ML 141 treatment group). **C.** ML 141 decreases HUVEC adhesion to fibronectin-coated surface. HUVEC were pretreated as above, lifted from culture dishes using cell scrapers, and replated (2 h) onto non-tissue culture treated plates that had been pre-coated with fibronectin and had been blocked with bovine serum albumin. Following extensive washes, adherent HUVEC were recovered using trypsin and counted using an Accuri C6 flow cytometer ($*P < 0.05$ by Student's t -test data pooled from 2 independent experiments, $n=8/\text{treatment}$).

Table 1

Molecular structure of ML 141 and related RSM pyrazoline analogs.

ID	Ar	R	Ar'	R'
ML 141	Ph	H	4-MeOPh	H
RSM 04	4-MeOPh	H	4-ClPh	H
RSM 05	Ph	H	3,4-(OCH ₂ O)Ph	H
RSM 06	4-MeOPh	H	3,4-(OCH ₂ O)Ph	H
RSM 07	4-MeOPh	H	Ph	H
RSM 11	Ph	H	4-MeOCH ₂ CH ₂ OPh	H
RSM 12	4-MeOPh	H	4-MeOCH ₂ CH ₂ OPh	H
RSM 13	Ph	H	3,4,5-MeOPh	H
RSM 14	4-MeOPh	H	3,4,5-MeOPh	H
RSM 15	Ph	H	3,4-MeOPh	H
RSM 16	4-MeOPh	H	3,4-MeOPh	H
RSM 17	4-MeOCH ₂ CH ₂ OPh	H	4-MeOPh	H
RSM 18	4-MeOCH ₂ CH ₂ OPh	H	4-MeOCH ₂ CH ₂ OPh	H
RSM 19	Ph	Me	4-MeOPh	H
RSM 20	4-MeOCH ₂ CH ₂ OPh	H	4-ClPh	H
RSM 21	Ph-(2-CH ₂)-		4-MeOPh	H
RSM 26	Ph	H	4-MeOPh	Me

Table 2

Assessment of ML 141 structural analogs (RSM 04-26) by invasion assay.

Internalized bacteria (% vehicle control $\pm$ SEM)		
RSM structural analog (10 μ mol/L)	ML 141 (10 μ mol/L)	
RSM 04	11 $\pm$ 1 % ^{*†††}	35 $\pm$ 5 % [*]
RSM 05	38 $\pm$ 3 % ^{*††}	42 $\pm$ 3 % [*]
RSM 06	23 $\pm$ 1 % ^{*†††}	39 $\pm$ 5 % [*]
RSM 07	34 $\pm$ 2 % ^{*††}	36 $\pm$ 3 % [*]
RSM 11	61 $\pm$ 2 % ^{*††}	57 $\pm$ 6 % [*]
RSM 12	57 $\pm$ 3 % ^{*††}	47 $\pm$ 4 % [*]
RSM 13	62 $\pm$ 5 % ^{*††}	63 $\pm$ 6 % [*]
RSM 14	71 $\pm$ 3 % ^{*†}	51 $\pm$ 3 % [*]
RSM 15	45 $\pm$ 1 % ^{*†††}	53 $\pm$ 3 % [*]
RSM 16	63 $\pm$ 4 % ^{*†††}	89 $\pm$ 3 % [*]
RSM 17	67 $\pm$ 8 % ^{*††}	66 $\pm$ 1 % [*]
RSM 18	76 $\pm$ 7 % ^{*†}	52 $\pm$ 3 % [*]
RSM 19	61 $\pm$ 2 % ^{*††}	58 $\pm$ 3 % [*]
RSM 20	61 $\pm$ 4 % ^{*††}	50 $\pm$ 14 % [*]
RSM 21	79 $\pm$ 3 % ^{*†}	61 $\pm$ 3 % [*]
RSM 26	40 $\pm$ 3 % ^{*††}	38 $\pm$ 3 % [*]

* N = 5; less than vehicle control;

† greater than ML 141;

†† not different than ML 141 ($P > 0.05$);††† less than ML 141, $P < 0.05$ by one-way ANOVA (post-hoc: Student-Newman-Keuls)

Table 3

Assessment of ML 141 and of structural analogs (RSM 04-26) by cytotoxicity and bactericidal assays.

	Mean fluorescence intensity ($\times 10^4 \pm \text{SEM}$)		CFU/ml ($\times 10^{10} \pm \text{SEM}$)	
	Vehicle control	Compound (10 $\mu\text{mol/L}$)	Vehicle control	Compound (10 $\mu\text{mol/L}$)
ML 141	1.7 $\pm$ 0.1	2.1 $\pm$ 0.3	2.0 $\pm$ 0.3	1.8 $\pm$ 0.6
RSM 04	1.9 $\pm$ 0.2	4.6 $\pm$ 0.2 *	1.2 $\pm$ 0.3	0.8 $\pm$ 0.2
RSM 05	2.6 $\pm$ 0.8	3.9 $\pm$ 0.2	2.4 $\pm$ 0.1	1.6 $\pm$ 0.3
RSM 06	2.4 $\pm$ 0.3	2.8 $\pm$ 0.1	0.7 $\pm$ 0.1	1.0 $\pm$ 0.3
RSM 07	1.5 $\pm$ 0.8	2.5 $\pm$ 0.4	2.3 $\pm$ 0.2	2.8 $\pm$ 0.3
RSM 15	3.6 $\pm$ 0.8	4.0 $\pm$ 0.4	1.7 $\pm$ 0.1	2.0 $\pm$ 0.2
RSM 16	1.9 $\pm$ 0.2	1.4 $\pm$ 0.2	1.7 $\pm$ 0.05	1.8 $\pm$ 0.4
RSM 19	2.0 $\pm$ 0.3	2.6 $\pm$ 0.01	2.1 $\pm$ 0.3	0.2 $\pm$ 0.9
RSM 20	2.6 $\pm$ 0.8	2.1 $\pm$ 0.2	2.5 $\pm$ 1.1	2.1 $\pm$ 0.4
RSM 21	2.4 $\pm$ 0.3	2.8 $\pm$ 0.09	2.9 $\pm$ 0.3	3.0 $\pm$ 0.2
RSM 26	13.3 $\pm$ 1.5	26.8 $\pm$ 2.1 *	2.0 $\pm$ 0.2	1.2 $\pm$ 0.7 *

* N = 3; different than vehicle control, $P < 0.05$ by Student's *t*-test ; CFU, colony forming unit