

CID 2950007 AS AN INHIBITOR OF  
*STAPHYLOCOCCUS AUREUS* INFECTIONS

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## List of Abbreviations

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)

Cell division cycle (CDC)

Dimethylallyl diphosphate (DMAPP)

Dimethyl sulfoxide (DMSO)

Farnesyl pyrophosphate (Fpp)

Geranylgeranyl pyrophosphate (GGpp)

GTPase-activating proteins (GAPs)

Guanine diphosphate (GDP)

Guanine-nucleotide exchanging factors (GEFs)

Guanine triphosphatases (GTPases)

Guanine triphosphate (GTP)

Human embryonic kidney (HEK)

Immunoprecipitation (IP)

Isopentenyl diphosphate (IPP)

Mean fluorescence intensity (MFI)

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Phosphoinositide (PI)

Phosphoinositide-3 Kinase (PI3K)

Phosphoinositide 3,4,5-triphosphate (PIP3)

Rho-GTPase-activating protein (RhoGAP)

*Staphylococcus aureus* (*S. aureus*)

Systemic inflammatory response syndrome (SIRS)

## Introduction

### Staphylococcus aureus and Sepsis

*Staphylococcus aureus* (*S. aureus*) infections are deleterious to the body, creating widespread infection, sepsis, blood coagulation, and even death [1]. *S. aureus* is a Gram-positive cocci bacterium that is part of the Micrococcaceae family. *S. aureus* infections are easily contracted in hospitals during surgery or simply during an extended stay for treatment. The number of *S. aureus* infections has risen significantly over the past twenty years. While some infections are contracted outside of the hospital, many are not. Hospitals remain the main locations where many infections begin. Another noticeable trend has been the increase of methicillin-resistant *S. aureus* (MRSA) strains that infect at-risk hospital patients and can be contracted through community-acquired infection as well. The death rate from *S. aureus* infections is estimated at 11-43% and has remained in that range for fifteen years.

There are a number of diseases that are caused by *S. aureus* infections. In addition to bacteremia, the number of cases of endocarditis caused by a *S. aureus* infection has risen [1]. Invasive procedures such as bone replacements and intravascular catheters put patients at the highest risk of contracting a bacterial infection. *S. aureus* also has a tendency to metastasize to the bones, joints, kidneys, and lungs, making recovery longer and more arduous. Other complications such as toxic shock syndrome and sepsis can also follow, again making recovery difficult.

Current treatments to combat a *S. aureus* infection include treatment with penicillin, vancomycin, or a combination of antimicrobials [1]. Penicillin remains the top choice if the bacterial strain has not developed resistance to this antibiotic, but this is rare. If resistance has been established, vancomycin is the best option. However, a few studies have suggested that a combination of antimicrobials may be best in combatting a bacterial infection and in clearing the bacteria from the body. Antimicrobial regimens and treatment times are variable, but anywhere from two to four weeks may be required for full treatment and recovery. While certain prophylactic measures can be taken to avoid contracting a *S. aureus* invasion, the best overall principle is to implement the appropriate procedures of infection control in hospitals.

The incidence of a bacterial infection leading to sepsis is about 33% [2]. But since 1987, Gram-positive bacteria have been the most common bacteria leading to severe sepsis, with the majority of cases caused by *S. aureus*. Recent estimates show that about 48% of the cases of severe sepsis are linked to Gram-positive bacterial infections. The development of methicillin-resistant *S. aureus* has added to this high percentage. Gram-negative infections remain the second most common bacterial cause of sepsis. Both fungal and viral infections can also lead to sepsis.

Sepsis is a threat to people infected with *S. aureus*, and the number of sepsis-related deaths in the US was over one million in the six year period from 1999-2005 [3]. The percentage of cancer patients who die from sepsis has reached close to 10% [4]. Demographics that are more susceptible to sepsis include the elderly, along with those suffering from diabetes, HIV infections, cancer, and alcohol abuse [2]. Symptoms such as tachycardia, high or low body temperature, high respiration rates, and either low or

high white blood cell counts are associated with systemic inflammatory response syndrome (SIRS). SIRS can progress to the designation of sepsis when a source of infection has been identified. If multiple organ dysfunction ensues, then a state of severe sepsis has been reached. The most commonly affected organs are the kidney, lung, and heart. Septic shock occurs when a patient is in severe sepsis along with cardiovascular dysfunction. These patients usually require vasopressin to maintain a normal blood pressure, and extended hospital stays are likely. Therapeutic treatments have been developed aggressively, mainly due to the increased number of patients infected with *S. aureus* [1] and the annual cost of treatment for these patients, which has reached about \$16 billion in the United States alone [5].

### Statins

The development of drugs to combat high cholesterol stretches back many years, but the mid 1970s is usually considered the beginning of the history of statins. Drugs known as compactin and lovastatin were first tested in the late 1970s on a number of different animals [6]. Positive results in these animals led to human trials in 1980. However, both drugs were soon removed from clinical trials, as their cytotoxicity in animals was brought into question. These suspensions were removed over the next couple of years, and lovastatin trials resumed in 1984.

Following this, lovastatin became available as a prescription for those with high cholesterol [6]. Variations on lovastatin such as pravastatin and simvastatin were developed over the next several years. Unequivocal mitigation in mortality rates associated with high cholesterol was seen in the years that followed. Statins have since been established as an efficacious drug to lower cholesterol. In recent years, though, an

unexpected side effect has been observed. Surprisingly, people who were taking statins prior to an acute bacterial infection responded much better to the infection, often times able to avoid becoming septic [7-9]. Therefore, statins continue to be tested in clinical trials as drugs protective from sepsis [clinicaltrials.gov].

Statins lower cholesterol [6]. They work by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is an enzyme in the cholesterol biosynthesis pathway. Consequently, all intermediates following this enzyme in the pathway are diminished, such as mevalonate and isoprenoid intermediates [10]. HMG-CoA reductase is not a product of the pathway; rather, it is an enzyme in the cholesterol-producing process. There is a distinct possibility that the protection of infected patients comes from the inhibition of this step in the pathway [11]. Therapeutic or even prophylactic approaches could be designed using statins. This way, patients entering a hospital for surgeries such as hip replacements or other invasive procedures would have a decreased chance of contracting a *S. aureus* infection.

However, concerns have been expressed over the use of statins in severely ill patients [12-16]. The main concern is that the mitigated amount of mevalonate deleteriously affects proper mitochondrial function in septic patients [16]. Ubiquinone is an important antioxidant in mitochondrial respiratory function, and the depletion of mevalonate results in the depletion of ubiquinone. This has caused researchers to search for alternative drugs or compounds that could serve as an acceptable substitute for statins.

#### Isoprenoid Intermediates

Isoprenoid intermediates in the cholesterol biosynthesis pathway play a role in protein-protein interactions, and they also serve as membrane anchors [10]. Isoprenoids

are made in all living beings [17]. In humans, farnesyl pyrophosphate (Fpp) and geranylgeranyl pyrophosphate (GGpp) are synthesized downstream of mevalonate in the cholesterol production pathway. The structure of isoprenoids consists of five-carbon chain units, known as isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Specifically, Fpp is a 15-carbon chain of lipids, but GGpp is a 20-carbon lipid chain. Both of these isoprenoids modify proteins after translation. They do this by binding to a cysteine residue. This process is known as prenylation. This modification role is mainly used with proteins containing the basic motif of CaaX [18]. Here, C indicates a prenylated cysteine residue, a indicates an aliphatic amino acid, and X is an amino acid determining whether GGpp or Fpp transferases are used for recognition [19]. This prenyl is a hydrophobic chain attaching the now prenylated protein to the cell membrane, resulting in re-localization of certain proteins to the membrane [17]. One class of proteins that contains this CaaX motif is the small G-proteins.

### G-proteins

Small G-proteins are typically referred to as small guanine triphosphatases (GTPases). Small G-proteins form a superfamily of proteins that play roles in cytoskeleton rearrangement, which can lead to endocytosis [17]. Every GTPase alternates between the active state and the inactive state based on how it is bound [20]. When bound to guanine diphosphate (GDP), GTPases are inactive; when bound to guanine triphosphate (GTP), GTPases are active. The two proteins that aid in this switch are guanine-nucleotide exchanging factors (GEFs) and GTPase-activating proteins (GAPs). GEFs allow the transformation from inactive to active, and GAPs aid with GTPases returning to the inactive state of being GDP-bound.

The Rho subfamily of GTPases includes Rho, Rac, and cell division cycle (CDC) 42 [20]. These GTPases play a role in cytoskeletal rearrangement [21], and they influence protein interactions between the membrane and the cytosol. It has been shown that treatment with statins decreased the rate of prenylation of these small GTPases [22]. If no prenylation can occur, then it logically follows that proteins cannot be bound to the cell membrane by prenylation. The absence of isoprenoid intermediates such as Fpp and GGpp may influence the decreased rate of prenylation of these GTPases.

#### Phosphoinositide-3 Kinase (PI3K)

PI3K is a kinase that phosphorylates the 3' position of a phosphoinositide, also playing a role in cytoskeletal rearrangement [23]. The specific isoform under investigation in this thesis work is p85, which is a regulatory subunit of the class PI3K [24]. There is also a catalytic subunit known as p110. When PI3K approaches the cell membrane, the p85 subunit can interact with many proteins, including small GTPases such as CDC42. The catalytic subunit p110 can also begin interacting with this CDC42/p85 complex, contributing to a possible mechanism of infection of *S. aureus*.

#### Mechanism of Infection

The mechanism by which *S. aureus* invades host cells is one of exploitation. The proposed mechanism of invasion has been postulated through many experimenters over many years. *S. aureus* expresses a protein known as fibronectin-binding protein, which allows *S. aureus* to bind to a host cell protein [25]. Once the fibronectin is bound, it initiates interaction with the  $\alpha_5\beta_1$  integrin at the cell surface of the host cell [26]. In response to *S. aureus* binding to fibronectin and interacting with the  $\alpha_5\beta_1$  integrin, the protein p85 attaches to CDC42 through the Rho-GTPase-activating protein (RhoGAP) domain of p85 [27]. Another factor influencing the formation of this CDC42/p85

complex is postulated to be the GTP-loading of CDC42 that occurs in the presence of *S. aureus* invasion [28]. Also included in this CDC42/p85 complex is the catalytic subunit p110. The regulatory subunit p85 serves as a link between p110 and the membrane-bound protein, in this case CDC42 [29]. The p110 catalytic subunit phosphorylates membrane-bound phosphoinositides. One of these is phosphoinositide 4,5-biphosphate, forming phosphoinositide 3,4,5-trisphosphate (PIP3) [24]. It is postulated that PIP3 then binds to  $\alpha$ -actinin, disrupting actin dynamics [30], and potentially this action brings in the complex containing the *S. aureus*.

Statins affect this particular process of infection by altering membrane localization of CDC42. This membrane localization is altered by the decrease in the intermediates in the cholesterol biosynthesis pathway [6]. Specifically regarding the effects of statins on *S. aureus* infections, the intermediates affected are the isoprenoids GGpp and Fpp [10]. It was shown that *S. aureus* invasion was restored following the replenishing of GGpp or Fpp following treatment with simvastatin [11]. This result suggested that it is the depletion of the isoprenoid intermediate that plays a major role in inhibition of invasion. It was also shown that small GTPases accumulated in the cytosol, including CDC42, in response to simvastatin treatment.

Horn *et al* then postulated the following, after extensive experimentation. Statins inhibit *S. aureus* invasion through the sequestration of PI3K (p85) plus its associated GTPases in the cytosol [11]. These GTPases are CDC42, and possibly Rac and RhoB. This sequestration occurs because the isoprenoid intermediates are not in sufficient quantity to bind to CaaX motif-containing proteins. Catalytic domains of PI3K that rely upon these small-GTPases to gain access to the membrane where PI resides are

therefore not able to come into contact with the PI. PIP3 formation therefore is limited as is its interaction with  $\alpha$ -actinin. In this way, the disassembly of the actin stress fibers is limited, and the fibronectin-bacteria complex remains on the cell surface. Through this proposed mechanism, simvastatin inhibits host invasion by restricting CDC42 and other small GTPases within the cytosol.

#### CID 2950007

A compound known as CID 2950007 has been recently shown to have similar effects to those of statins. Preliminary experiments performed by Surviladze *et al* [31] have shown that CID 2950007 inhibits CDC42 activity by binding to it and inhibiting GTP-loading. Surviladze *et al* were able to show that CID 2950007 is highly selective for CDC42 and not for any other member of the GTPase family that was tested. Further testing revealed a mechanism of allosteric control between CID 2950007 and CDC42. Once CID 2950007 binds to the allosteric site on CDC42, GTP cannot bind to it. The postulation therefore is that if GTP cannot bind to CDC42, then CDC42 cannot properly associate with p85. Therefore, PIP3 formation is reduced, decreasing the interaction between PIP3 and  $\alpha$ -actinin. Actin stress fibers remain static, and the bacteria is not taken in through endocytosis.

Preliminary data, therefore, suggest that this compound CID 2950007 may be able to prevent *S. aureus* infection through a different mechanism than statins. While statins restrict CDC42 to the cytosol, CID 2950007 inhibits CDC42's activity by binding to it directly. If CID 2950007 truly can offer the same protection as statins while evading the more deleterious effects associated with decreasing the intermediates in the cholesterol

biosynthesis pathway, then CID 2950007 could possibly be used as a more efficacious alternative to statin use as a means of protection against sepsis.

### Significance

*S. aureus* is one of the most infectious bacterium in the United States today [1]. Infections occur when bacteria enter the body through a breach in the skin. Complications can soon arise. These include bacteremia; endocarditis; metastatic invasion to the joints, kidneys, and lungs; and sepsis. The abundance and proliferation of MRSA has also contributed to the growing concern surrounding sepsis. Those with already-compromised immune systems or the elderly are at risk to develop severe complications following the onset of an acute *S. aureus* infection [2].

One of the complications arising from infections is sepsis, which occurs more in the winter months and among those who already had health problems prior to infection [2]. In 2008, sepsis was the 10<sup>th</sup>-leading cause of death in the United States [32], and there were 1,017,616 deaths between 1999 and 2005 associated with sepsis. Death rates due to sepsis have been continuously growing over the last two decades [33], especially among at-risk patients who are already ill. The estimated annual cost to treat septic patients has reached \$16.7 billion [5]. Most often, the cause of death in a septic patient is the failure of multiple organs [34]. While the rise in septic-related deaths has slowed somewhat in the last few years, the growing population of elderly in the country has added to the continuously growing number of at-risk patients [3]. The development of adjunctive therapies has therefore been on the rise in the last several years in an attempt to combat the growing number of septic-related deaths [1].

One such proposed therapy is the use of statins, which have been shown to provide protection from *S. aureus* infections [7]. Statins work by inhibiting the production of HMG-CoA reductase, which is an enzyme in the cholesterol biosynthesis pathway. This inhibition leads to the lowering of isoprenoid intermediates, such as mevalonate, GGpp, and Fpp. It is postulated that since these isoprenoid intermediates are not available to bind to the proteins associated with *S. aureus* uptake, intracellular infection rates are abated [11].

However, concerns have been expressed regarding the use of statins in the critically ill [12-16]. The main concern is that the diminished amount of mevalonate deleteriously affects proper mitochondrial function in septic patients. Ubiquinone is an important antioxidant in mitochondrial respiratory function, and the depletion of mevalonate results in the depletion of ubiquinone. An associated risk is that low ubiquinone levels are associated with both rhabdomyolysis and myopathy.

An alternative approach has been investigated. The compound CID 2950007 has been shown to act specifically on and block CDC42 [31]. This inhibition has the same effects as statins in terms of protection against *S. aureus* invasion, but CID 2950007 functions independently of mevalonate. The hypothesis is therefore that there is no depletion of isoprenoid intermediates, potentially reducing associated risks. CID 2950007 inhibits GTP-loading on CDC42 with high specificity. Rather than disrupting membrane localization through an indirect mechanism, as statins do, CID 2950007 acts much more directly. If continual positive outcomes are seen *in vitro* and *in vivo*, then this compound might prove to be a valuable alternative to statin use in the critically ill. CID

2950007 would provide the same protection against *S. aureus* invasions that statins do, while circumventing the more deleterious effects.

Purpose

The goal of this thesis was to examine whether CID 2950007, serum starvation, and DMSO is cytotoxic to cells; whether CID 2950007 inhibits invasion in different doses in HEK293 cells; and whether CID 2950007 does indeed function by inhibiting the CDC42/p85 association directly. The goal was to provide evidence that supports these theories, which would indicate that CID 2950007 should be considered as an adjunctive therapy for those at risk of developing *S. aureus* infections and subsequently becoming septic.

## **Hypothesis**

CID 2950007 inhibits *S. aureus* invasion of host cells through a non-cytotoxic mechanism.

## **Research Methods**

### Immunoprecipitation (IP)

IP is a method used to isolate one protein from a large number of mixed proteins. This is done by first incubating with a primary antibody that will attach to the desired protein antigen. Following this, a solid substrate (Protein A, for instance) would be used to precipitate both the antibody and the attached antigen. This method allows the precipitation of just one desired protein amongst a conglomeration of many.

### Flow Cytometry

Cytometry is a process by which characteristics of cells are measured; other small particles, such as vesicles, can also be measured this way. In flow cytometry, these cells are measured or counted as they flow in a single line. In this particular study, *S. aureus* bacteria were fluorescently tagged with a fluorophore that excites at a particular wavelength. When cells were sent through the flow cytometer, those that were infected with *S. aureus* were counted. This counting occurred due to the excitation of the fluorophore as it passed through the sensor in the flow cytometer. This way, a percentage of the infected cells could be obtained, along with total mean fluorescence intensity (MFI) for the sample as a whole. This method was the predominant one used in this study due to its lack of variability when compared to the previous method of plating serial dilutions of infected cells on tryptic soy agar plates and manually counting the number of colonies on each.

### Cell lines

HEK293: These are human embryonic kidney cells [35]. These adherent cells were first developed in the 1970s, and are still widely used today. They grow well in standard tissue culture conditions (37° C, 5% CO<sub>2</sub>), but are tumorigenic [American Type Culture Collection (ATCC), Manassas, VA]. They are rated biosafety level 2 since they do contain adenovirus. They were originally transformed with a left-end sequence of the adenovirus DNA, a viral genome [36]. This DNA was incorporated into the host DNA. HEK293 cells appear small and circular in morphology [ATCC]. Their ideal medium consists of DMEM, FBS, and L-glutamine.

U87-MG: U87 cells are adherent and rated biosafety level 1 [ATCC]. These cells were obtained from a malignant glioblastoma several years ago [37]. U87-MG cells are large and elongated in morphology [ATCC]. They are quite tumorigenic, and are best grown in 37° C and 5% CO<sub>2</sub>. Their medium includes DMEM, FBS, NEAA, and sodium pyruvate.

*Culture of Cells* HEK cells (#CRL-1573, ATCC) were grown in DMEM (#11960-044, Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (S11150, Atlanta Biologicals, Norcross, GA) and L-glutamine (#25030-081, Invitrogen) (5% CO<sub>2</sub>, 37°C) in 75-cm<sup>2</sup> vented cap flasks (#430641, Fisher Scientific, Pittsburgh, PA) before plating. For the western blot assay, cells were plated at 5X10<sup>5</sup> cells/mL; for all other assays, 3X10<sup>5</sup> cells/mL. Cells were plated on 100 mm tissue culture dishes (#S-831802, Fisher Scientific) for the western blot assay, and on 35 mm tissue culture dishes (#08-772A, Fisher Scientific) for the other assays. Both plates were coated with Attachment Factor (#S-006-100, Invitrogen) prior to plating. CID 2950007 treatments were introduced on

day 3 of plating. For initial experiments, dosage was a 10  $\mu\text{M}$  concentration. As part of subsequent troubleshooting, dosage was as a 10  $\mu\text{M}$  concentration in 1% dimethyl sulfoxide (DMSO; #BP231-1, Fisher Scientific) to enhance solubility.

*Serum Starvation* Serum free media for HEK cells was made with DMEM plus L-glutamine. Tissue culture plates were washed gently with phosphate buffered saline (PBS; #20012-043, Invitrogen). Serum free media was gently added to each plate.

*Invasion Assay* Invasion was performed on day 3 of plating. *S. aureus* (#29213, ATCC) was subcultured in tryptic soy broth (TSB; #22092, Sigma, St. Louis, MO) (200 rpm, 37°C). Bacteria were harvested through centrifugation (10,000 rpm, 3 min, 37°C). The bacteria were washed once with 0.85% saline, and resuspended in saline at  $3 \times 10^8$  cells/mL. In experiments when the drug was added under serum free conditions, *S. aureus* was incubated with FBS for 15 minutes (room temperature), then washed and resuspended in saline as above. In this way, the *S. aureus* was coated with fibronectin, which is required for invasion. For experiment using flow cytometry, Alexa Fluor 488 rabbit anti-mouse IgG (#A11059, Invitrogen) was incubated along with the FBS. HEK cells were incubated with *S. aureus* (1 hr, 5%  $\text{CO}_2$ , 37°C). Invasion was arrested by the addition of lysostaphin (20  $\mu\text{g}/\text{mL}$ ) (#L7386, Sigma) and gentamicin (50  $\mu\text{g}/\text{mL}$ ) (#G1272, Sigma) which destroyed any extracellular bacteria (45 min, 5%  $\text{CO}_2$ , 37°C). Intracellular bacteria were freed from the cells and released into suspension by the addition of 1% saponin (20 min, 5%  $\text{CO}_2$ , 37°C). Serial dilutions of each medium were plated on tryptic soy agar (TSA; #22091, Sigma) plates and incubated (20 hours, 37°C). Colony counts were performed. When a fluorescent tag was used, the number of infected cells was counted using flow cytometry.

*Cell lysis* U87-MG cells (#HTB-14, ATCC) were plated on 100 mm tissue culture dishes using DMEM supplemented with FBS, non-essential amino acids (NEAA; #11140-050, Invitrogen), and sodium pyruvate (#11360-070, Invitrogen). Cells were plated at  $5 \times 10^5$  cells/mL. The cells were pre-treated with CID 2950007 or DMSO on day 3, followed by bacterial invasion (MOI 100), all performed as above, but not serum free. Plates were kept on ice, aspirated, washed once with ice cold PBS, aspirated again, and treated with RIPA lysis buffer (50 mM HEPES (#15630080, Invitrogen), 150 mM NaCl (#MSX04251, Fisher Scientific), 1.5 mM  $MgCl_2$  (#EM-5980, VWR, Radnor, PA), 1 mM EGTA (#E3889, Sigma), 10% glycerol (#15514-011, Invitrogen), 1% Triton X-100 (#T8787, Sigma), 1% Na deoxycholate (#D6750, Sigma), 0.1% SDS (#L4509, Sigma), 1 Mini-tab (#1836153, Roche, Indianapolis, IN), pH 7.4). Cells were scraped, rocked (4°C, 20 min), and centrifuged (10000g, 4°C, 10 min). The supernatants were stored in cold tubes and contained cytosolic proteins. Total protein concentration was determined using the Bio-Rad Protein Assay (#5000006, BIO-RAD, Hercules, CA).

*Immunoprecipitation* To isolate CDC42 from the other cytosolic proteins, cell fractions from each plate were incubated overnight (rocking, 4°C) with anti-CDC42 (#610928, BD Biosciences, Bedford, MA). The immunocomplex was recovered using Protein A (#15918-014, Invitrogen). Extensive washing with both ice cold PBS and ice cold LiCl buffer (500 mM LiCl (#LK4408, Sigma) /100 mM Tris-HCl (#JM-2107-100, MBL International, Woburn, MA), pH 7.5) followed. The immunocomplex was then retrieved by boiling in LDS-Sample buffer (#NP0007, Invitrogen) with reducing agent (#NP 0009) (5 min) followed by a spin (3 min, 10,000g).

*Western Blot Assay* Both post- and pre-IP samples were subjected to electrophoresis using 4-12% NU-PAGE gel (#NP0321, Invitrogen) with 1X running buffer made from MES running buffer (#NP0002, Invitrogen). The proteins from the gel were transferred to a membrane using transfer buffer (#NP0006-1, Invitrogen). The blot was then blocked with Odyssey blocking buffer (#927-40003, LI-COR, Lincoln, NE) (room temp, 1 hour, shaking) and probed with blocking buffer/0.1% Tween 20 (#P5927, Sigma) along with anti-p85 (#06-195, Upstate, Lake Placid, NY) (overnight, 4°C). Membranes were then washed in PBS/0.1% Tween 20, then incubated with blocking buffer, 0.1% Tween 20, anti-rabbit 800CW (#R11-131-122, Rockland, Canada) (room temperature, 1 hour). The membrane was then washed in 1X PBS/0.1% Tween 20, and fluorescence at 800 was detected using the Odyssey Infrared Imaging System (LI-COR).

*Cytotoxicity Assay using Propidium Iodide* HEK cells were pretreated and invaded as above and at the concentrations in the figure legends. Cytotoxicity was assessed by examining the uptake of propidium iodide (PI, #P4170, Sigma) in PBS. Flow cytometry was used to assess the number of PI<sup>+</sup> cells.

*Cytotoxicity Assay using XTT* HEK293 cells were pretreated and invaded as above and at the concentrations in the figure legends. Cytotoxicity was assessed by examining the absorbance of wells in a 96-well plate following treatment with 0.5% menadione (#95034-382, VWR) in XTT (#89138-264, VWR). Absorbance was measured using a plate reader at a wavelength of 490 nm.

*Statistical Analyses* Normally distributed data for two groups were compared using Student's t-test. Non-normally distributed data for more than two groups were transformed by a log<sub>10</sub> function, and then analyzed using one-way ANOVA followed by

Student's Newman-Keuls post hoc analysis. All differences were considered significant at the  $p \leq 0.05$  level.

## Results

*Cytotoxicity was not detected in response to CID 2950007 treatment.*

In serum free conditions, CID 2950007 was shown to be non-cytotoxic to cells (figure 1) even in the midst of invasion (figure 2). Since the number of dead cells seemed elevated even in the control, further cytotoxicity analyses were performed. Through XTT analysis, no cytotoxicity was detected with DMSO treatment, but cytotoxicity was detected during serum starvation (figure 3). This indicated that the serum starvation contributed to the high rate of cell death in the previous experiments. The cytotoxicity assay was run again with CID 2950007, but this time using cells in complete media. XTT analysis was run again, and no cytotoxicity was detected following a 10  $\mu$ M dose of CID 2950007 given to cells supplemented with complete media (figure 4).

*CID 2950007 prevents *S. aureus* invasion.*

Inhibition of invasion was shown in both U87-MG cells (figure 5, work done by Diana Cordero, summer 2011) and in HEK293 cells. For HEK293 cells, both serum free conditions (figure 6) and complete media conditions (figure 7) were examined. Complete media conditions were examined after establishing that CID 2950007 did not cause any detectable cytotoxicity in complete media (figure 4). The promising results shown in figure 7 led to the question of whether a lower concentration of CID 2950007 would

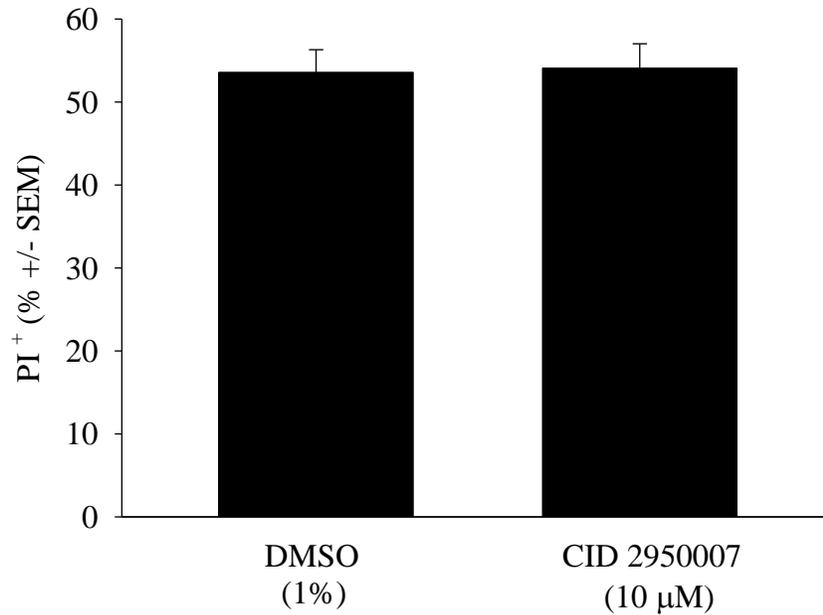
sustain inhibition of invasion. A 1  $\mu$ M dose of CID 2950007 was indeed shown to inhibit invasion, but with a higher number of replicates needed than usual (figure 8).

*CID 2950007 may prevent invasion through disruption of the CDC42/p85 complex.*

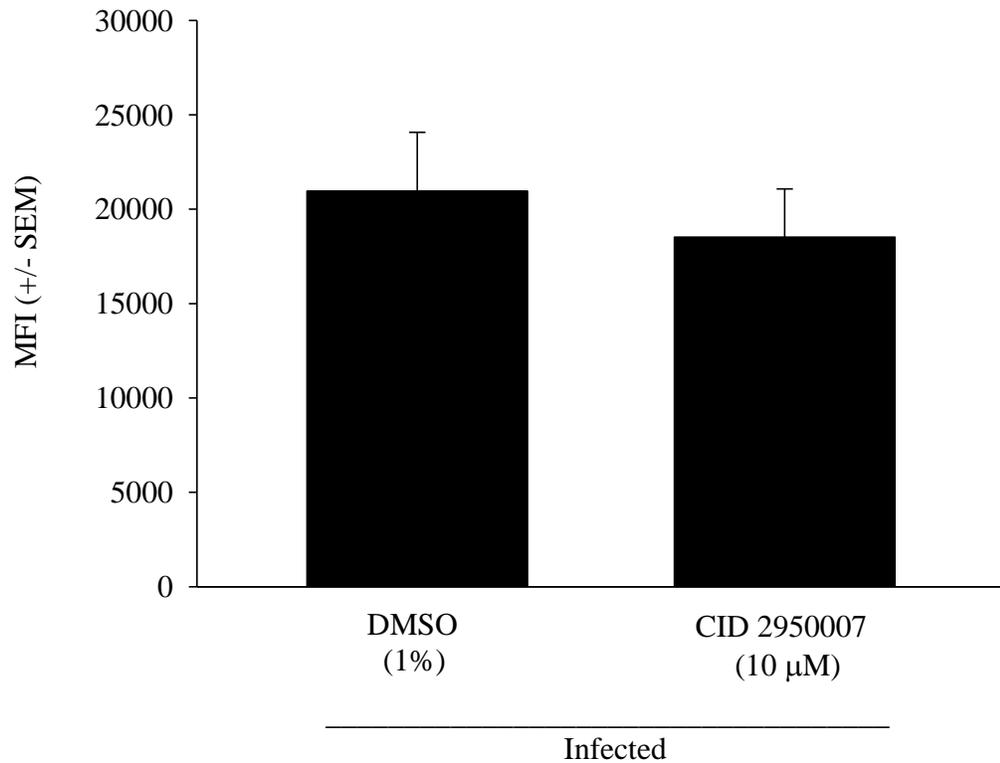
Western blot analysis in U87-MG cells provided tenuous results (figure 9), implying that the U87-MG cells did not respond well to the treatment. The p85 band was not present where it should have been. A decision was made to move onto HEK293 cells, as these had been shown in the past to respond well to this assay. When this experiment was performed in the past with HEK293 cells and infection, the CDC42/p85 association had been clear. Since the protocol for this experiment was the same except for the CID 2950007 treatment and the cell line, a decision was made to switch over to HEK293 cells in hopes that if CID 2950007 did provide protection for them just as it did in U87-MG cells, then the western blot assay would work as well.

The subsequent western blot showed promising results (figure 10), as bands can be seen in all lanes. The presence of bands in all lanes allows comparisons to be made between the untreated and treated groups; however, these bands were not quantified. Therefore, it is difficult to make visual comparisons. More blots should be performed with fresh antibody.

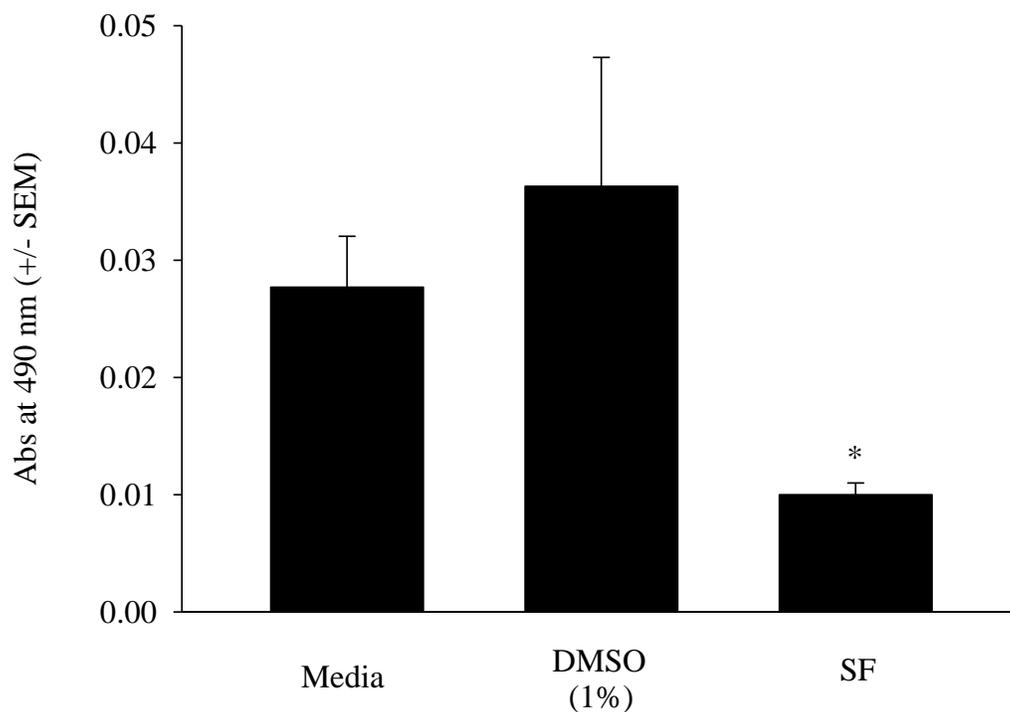
## Figures



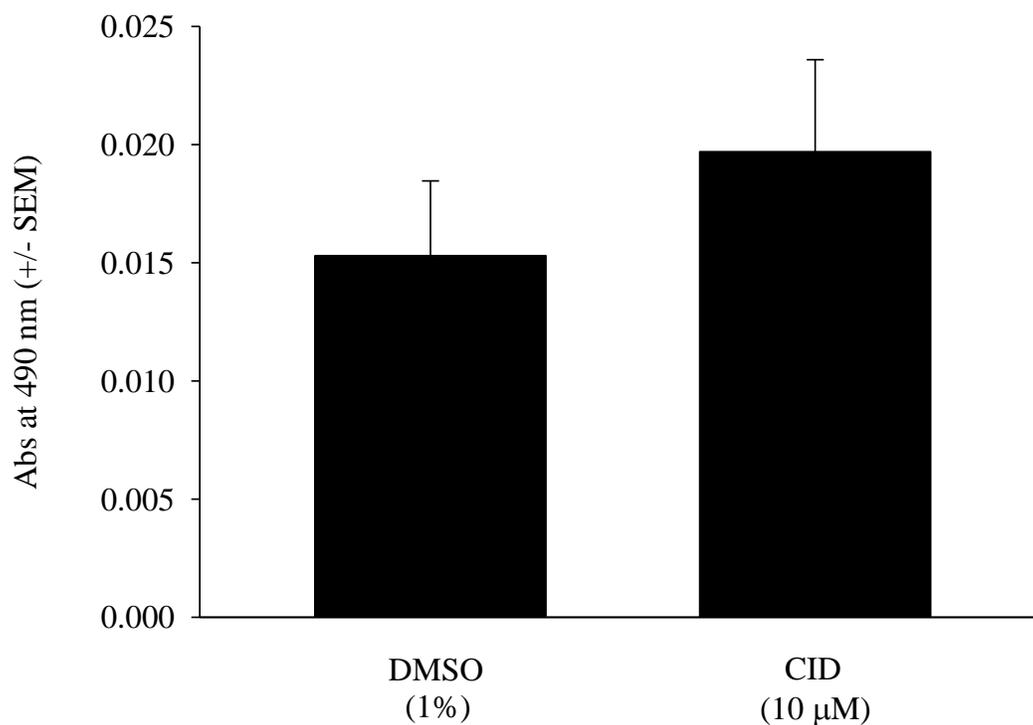
**Figure 1 Cytotoxicity is not detected in HEK293 cells treated with CID 2950007 in serum free media.** Cells were treated with CID 2950007 (10μM, 1 h) or dimethyl sulfoxide (DMSO, 1%, 1 h). Cytotoxicity was assessed by measuring propidium iodide (PI) uptake using flow cytometry. Data are presented as mean +/- SEM ( $p > 0.05$  by Student's t test).



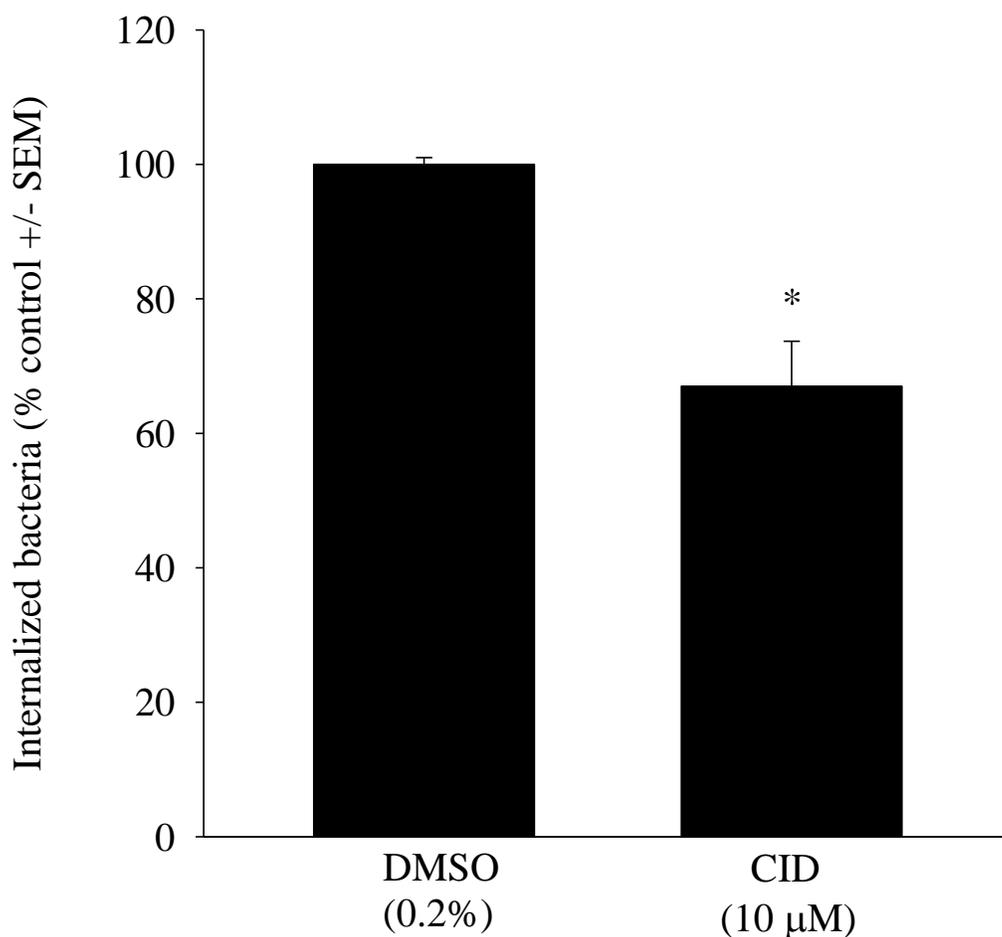
**Figure 2 Cytotoxicity is not detected in HEK293 cells treated with CID 2950007 during *S. aureus* invasion in serum free media.** Cells were pretreated with CID 2950007 (10μM, 1 h) or dimethyl sulfoxide (DMSO, 1%, 1 h) followed by *S. aureus* infection (multiplicity of infection 300, 1 h). Cytotoxicity was assessed by measuring propidium iodide uptake using flow cytometry. Mean fluorescence intensity (MFI) was measured. Data are presented as mean +/- SEM ( $p > 0.05$  by Student's t test).



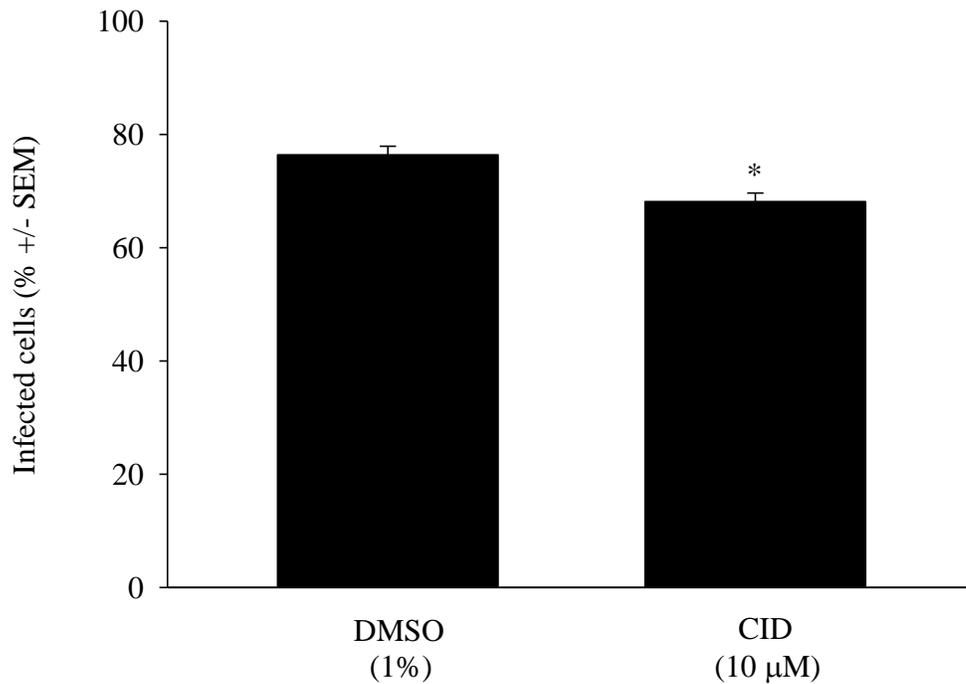
**Figure 3 Cytotoxicity is not detected in cells treated with DMSO given in complete media, but cytotoxicity is detected when cells are serum starved.** Cells were treated with either media alone, dimethyl sulfoxide (DMSO, 1%, 20 h) in complete media, or with media that was serum free (SF, 20 h). Cells were examined using XTT/menadione. Data are presented as mean +/- SEM (\*,  $p \leq 0.05$  compared to both media and DMSO treatment by one-way ANOVA followed by Student's Newman-Keuls post hoc analysis). Statistics were run on  $\log_{10}$  transformed data.



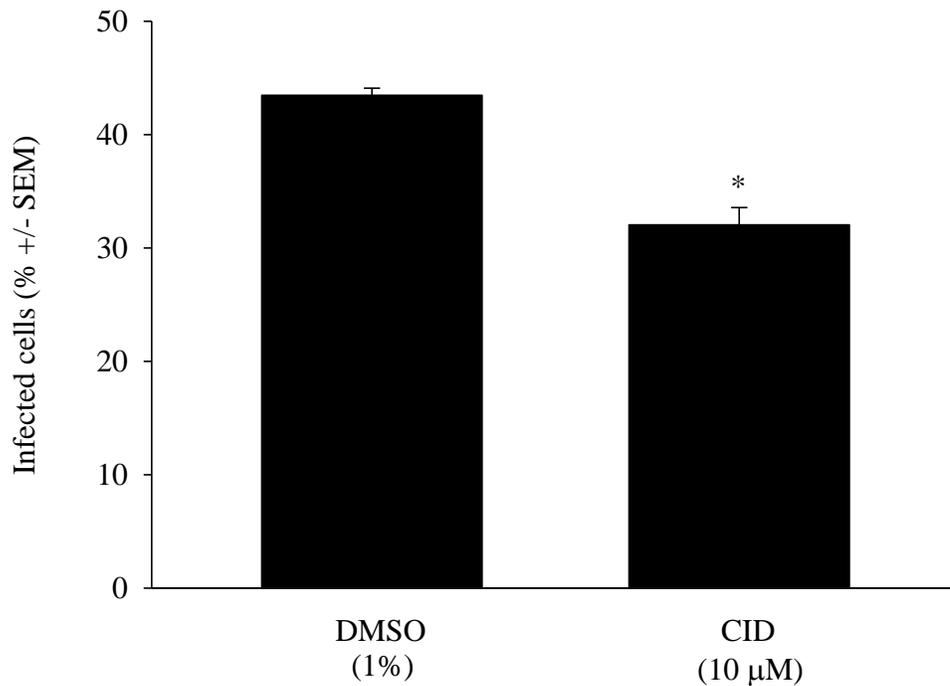
**Figure 4 Cytotoxicity is not detected in HEK293 cells treated with CID 2950007 in complete media.** Cells were treated with either DMSO (1%, 20 h) in complete media or CID 2950007 (10 μM, 20 h) in complete media. Cells were examined using XTT/menadione. Data are presented as mean +/- SEM ( $p > 0.05$  by Student's t test).



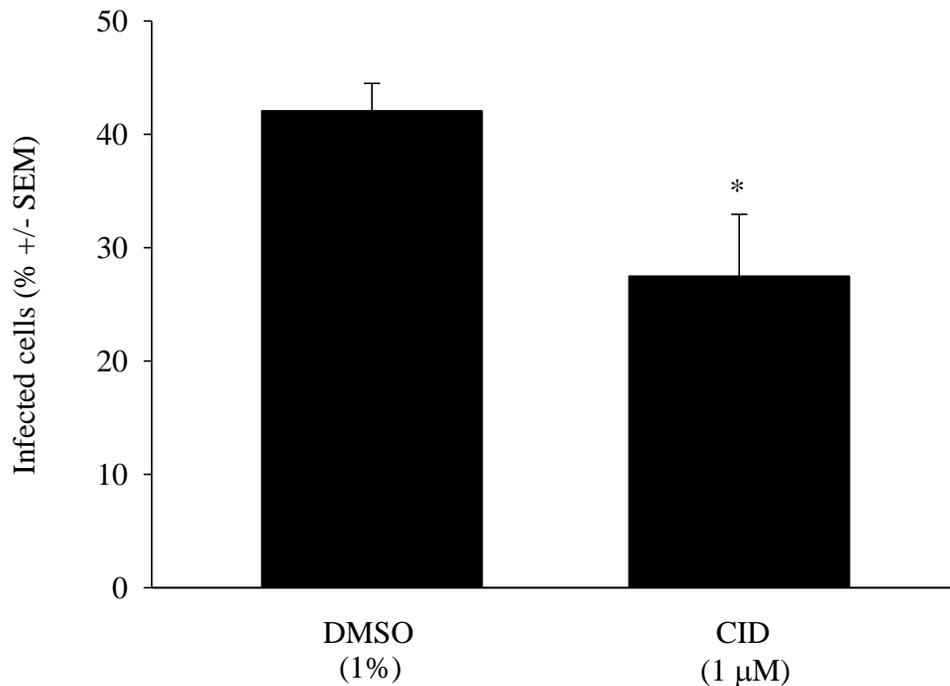
**Figure 5** CID 2950007 inhibits invasion of *S. aureus* in U87-MG cells. U-87MG cells were pretreated with CID 2950007 (10 μM, 1 h) or dimethyl sulfoxide (DMSO, 0.2%, 1 h) followed by *S. aureus* infection (multiplicity of infection 300, 1 h). Plates were treated with lysostaphin (50 μg/mL) and gentamicin (20 μg/mL) to remove extracellular bacteria. Intracellular bacteria were released with 1% saponin. Serial dilutions of the medium were plated on tryptic soy agar plates for colony counts (16 h). Data are presented as mean +/- SEM (\*,  $p \leq 0.05$  compared to DMSO control by Student's t test).



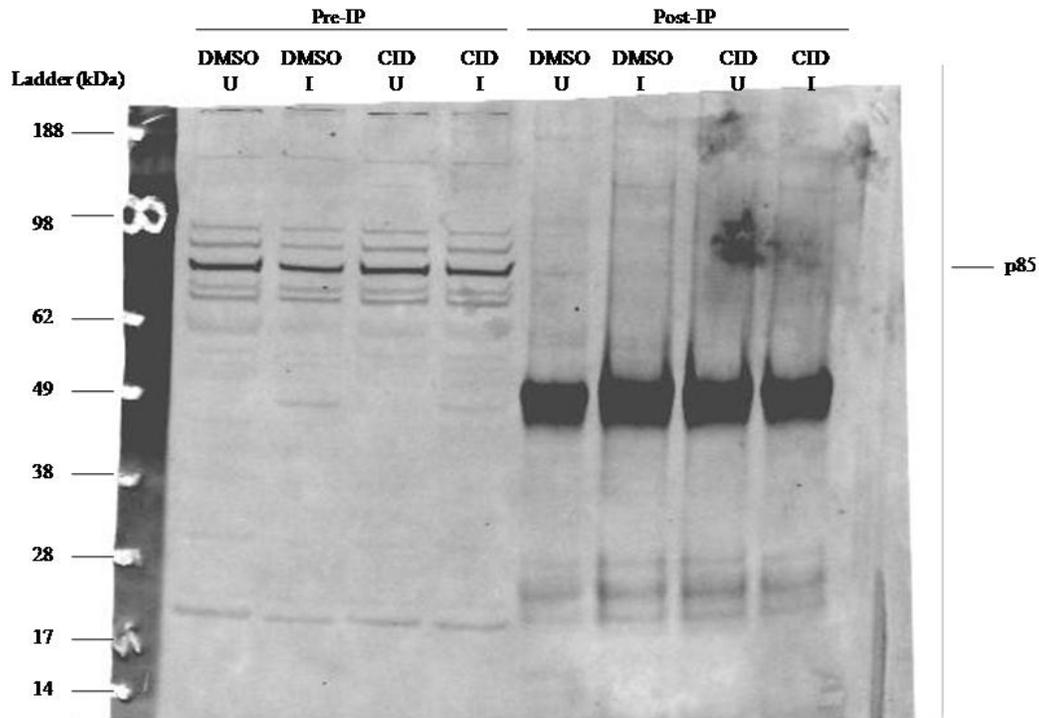
**Figure 6 CID 2950007 inhibits invasion of *S. aureus* in serum free conditions in HEK293 cells.** HEK293 cells were pretreated with CID 2950007 (10 μM, 1 h) or dimethyl sulfoxide (DMSO, 1%, 1 h). The *S. aureus* was fluorescently labeled, and cells were infected (multiplicity of infection 300, 1 h). Plates were treated with lysostaphin (50 μg/mL) and gentamicin (20 μg/mL) to remove extracellular bacteria. Flow cytometry was used to examine the number of cells that had taken up bacteria. Data are presented as mean +/- SEM (\*,  $p \leq 0.05$  compared to DMSO control by Student's t test).



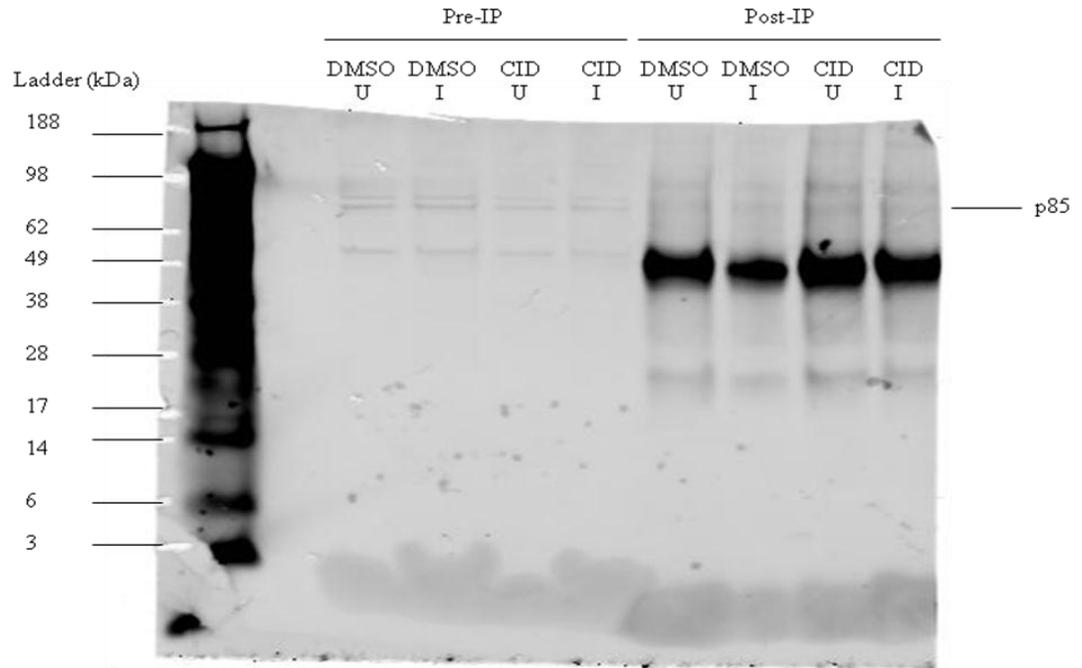
**Figure 7 CID 2950007 (10 μM) inhibits invasion of *S. aureus* in complete media.** HEK293 cells were pretreated with CID 2950007 (10 μM, 20 h) or dimethyl sulfoxide (DMSO, 1%, 20 h). The *S. aureus* was fluorescently labeled, and cells were then infected (multiplicity of infection 300, 1 h). Cells were treated with lysostaphin (20 μg/mL) and gentamicin (50 μg/mL). Flow cytometry was used to examine the percentage of cells that had taken up bacteria. Data are presented as mean +/- SEM (\*,  $p \leq 0.05$  compared to DMSO control by Student's t test).



**Figure 8 CID 2950007 (1 μM) inhibits invasion of *S. aureus*.** HEK293 cells were pretreated with CID 2950007 (1 μM, 20 h) or dimethyl sulfoxide (DMSO, 1%, 20 h). The *S. aureus* was fluorescently labeled, and cells were then infected (multiplicity of infection 300, 1 h). Cells were treated with lysostaphin (20 μg/mL) and gentamicin (50 μg/mL). Flow cytometry was used to examine the percentage of cells that had taken up bacteria. Data are presented as mean +/- SEM (\*,  $p \leq 0.05$  compared to DMSO control by Student's t test).



**Figure 9** The p85 band is not visible in the immunoprecipitation lanes, but is visible in the pre-immunoprecipitation lanes. U87-MG cells were pre-treated (1 hr) with dimethyl sulfoxide (DMSO) or CID 2950007 (CID, 10  $\mu$ M) and either infected (I) or not infected (U) with *S. aureus* (multiplicity of infection 100, 1 hr). Lysates were subjected to IP using anti-CDC42, and immunocomplexes were subjected to western blot analysis, and probed with anti-p85 followed by anti-rabbit 800CW.



**Figure 10** The p85 band is slightly visible in both the immunoprecipitation lanes and the pre-immunoprecipitation lanes. HEK293 cells were pre-treated (20 hr) with dimethyl sulfoxide (DMSO) or CID 2950007 (CID, 10  $\mu$ M) and either infected (I) or not infected (U) with *S. aureus* (multiplicity of infection 100, 1 hr). Lysates were subjected to IP using anti-CDC42, and immunocomplexes were subjected to western blot analysis, and probed with anti-p85 followed by anti-rabbit 680CW.

## Conclusion

Several cytotoxicity assays were run to examine whether the compound CID 2950007 was harmful to cells. No detectable cytotoxicity of CID 2950007 was detected in serum free media (figure 1). The same assay run in the midst of invasion also showed no detectable toxicity (figure 2). However, the number of dead cells was higher than expected even in the negative control. It was suspected that the serum free conditions were contributing to the high rate of cell death. Further testing revealed that it was the serum starvation contributing the most to the cell death (figure 3), while DMSO showed no detectable cytotoxicity. Given the impracticalities of serum free conditions, another cytotoxicity assay was run in complete media conditions, with CID 2950007 showing no detectable cytotoxicity in complete media conditions (figure 4). Together, these results suggest that CID 2950007 will not contribute to cell death in *in vivo* studies, where all cells are in natural serum conditions.

CID 2950007 has shown to be effective in inhibiting *S. aureus* invasion of host cells. CID 2950007 was previously shown to inhibit invasion in U87-MG cells (figure 5). Several experiments were run in HEK293 cells. Inhibition was sustained in serum free conditions (figure 6), but this again is impractical for real world applications. Following the establishment of the undetectable levels of cytotoxicity of CID 2950007, inhibition was sustained in a 10  $\mu$ M concentration (figure 7). Furthermore, a 1  $\mu$ M

concentration was also effective in inhibiting bacterial invasion (figure 8). These results altogether suggest that CID 2950007 is effective in providing protection from bacterial host invasion.

To attempt to examine the molecular mechanism of inhibition of invasion, two western blot assays were performed. The hypothesis of this research is that CID 2950007 prevents the binding of CDC42 to p85, disrupting the proposed mechanism of infection. The western blot of both the U87-MG cells (figure 9) and the HEK293 cells (figure 10) attempted to examine the relationship between the GTPase CDC42 and PI3K p85. The U87-MG cell western blot provided no clear results, but the HEK293 cells appeared to respond much better to the assay. A p85 band was present in all lanes. Non-specific binding suggests that fresh antibody should be used, but HEK293 cells will most likely respond well to this assay in future studies.

The working model of *S. aureus* invasion involves GTP-loading of CDC42 promoting its binding to p85 at the RhoGAP domain [11]. This occurs at the membrane in response to *S. aureus* binding to fibronectin and interacting with the  $\alpha_5\beta_1$  integrin at the cell surface of the host cell. Simvastatin was previously shown to inhibit invasion, but through the postulation that the depletion of isoprenoid intermediates restricted CDC42 to the cytosol, preventing its binding to p85 [11]. The current research examined the hypothesis that the compound CID 2950007 inhibited invasion through a more direct mechanism of binding to CDC42 and preventing GTP-loading. The postulation is that the inhibition of GTP-loading prevents the binding of CDC42 to p85.

CID 2950007 was shown to inhibit invasion in two different cell lines and in low concentrations; however, the western blots intended to illuminate the CDC42/p85

complex provided no clear evidence of the actual status of this complex. CID 2950007 has been shown to bind specifically to CDC42 [31], but no definite remarks can be made regarding the CDC42/p85 complex. What is clear is that CID 2950007 is providing protection from *S. aureus* invasion. A possible alternative explanation is that other GTPases, as of yet unidentified, are binding to p85 through the RhoGAP domain and playing a role in invasion. However, Horn *et al* [11] found that a mutation in the C of the CaaX motif of CDC42 reduced invasion by about 90%, suggesting that CDC42 is essential to the process of invasion. Therefore, other GTPases that may be contributing to the mechanism of invasion would likely have an inappreciable effect, and attention should remain focused on CDC42 since it is activated early during *S. aureus* invasion [38] and plays a role in actin dynamics [29].

Horn *et al* [11], in 2008, was able to show that Secramine A, a non-specific inhibitor of GTPases, significantly reduced invasion. However, the problem with Secramine A was its non-specificity. The promising aspect of this current research is that an inhibitor specific for CDC42 has been identified and been shown to inhibit invasion. Taken together, these results demonstrate that the compound CID 2950007 may prove to be useful in the development of therapeutic drugs for the resistance of *S. aureus* invasion leading to sepsis. A 10  $\mu$ M dose of CID 2950007 inhibits *S. aureus* invasion and is not cytotoxic to cells supplemented with complete media. This suggests that the same effect would be observed in *in vivo* studies. The status of the CDC42/p85 complex has yet to be revealed. The results of the western blot performed on HEK293 cells are promising. Using a fresh antibody would minimize the non-specific binding and would perhaps produce a better blot. A suggestion for the investigators who continue this research is to

begin with a 10  $\mu$ M dosage of CID 2950007 for the western blot assay, and then determine the appropriate next step based on those results. A more soluble form of the compound is under development, and this form should also be used in future studies. Now that cytotoxicity has been eliminated as a possibility and inhibition of invasion has been shown in complete media, the focus can shift to determining the status of the protein complex CDC42/p85 through western blot analysis.

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