UNIVERSITY OF WISCONSIN – LA CROSSE

Graduate Studies

INTRACELLULAR PENETRATION OF NOVEL ANTIMICROBIALS IN HUMAN THP-1 MACROPHAGES INFECTED WITH *LISTERIA MONOCYTOGENES*

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

Paula A. (Thomsen) Marsland

College of Science and Health
Clinical Microbiology

May, 2014

INTRACELLULAR PENETRATION OF NOVEL ANTIMICROBIALS IN HUMAN THP-1 MACROPHAGES INFECTED WITH *LISTERIA MONOCYTOGENES*

By Paula A. (Thomsen) Marsland

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the Degree of Master of Science in Biology, Clinical Microbiology Concentration.

The candidate has completed the oral defense of the thesis.

Marc A. Rott, Ph.D.
Thesis Committee Chairperson — 5/14/14 Date

William Schwan, Ph.D.
Thesis Committee Member — 5/13/14 Date

Aaron A. Monte, Ph.D.
Thesis Committee Member — 5/14/14 Date

Diane Sewell, Ph.D.
Thesis Committee Member — 5-9-2014 Date

Thesis Accepted:

Steven Simpson, Ph.D.
Graduate Studies Director — 5/30/14 Date

ABSTRACT

Marsland, P.A. (Thomsen) Intracellular penetration of novel antimicrobials in human THP-1 macrophages infected with *Listeria monocytogenes.* MS in Biology, Clinical Microbiology Concentration, May 2014, 77pp. (M.A. Rott)

Antimicrobial resistance is increasing world-wide and there is a need for new, more effective drug treatments. Intracellular bacterial infections are among the most deadly and difficult to treat. Members of the *Mycobacterium tuberculosis* complex infect intracellularly and are one of the top ten causes of death worldwide. Novel stilbenoid compounds have been identified that kill *M. tuberculosis in vitro*. Human macrophage cells were infected with *Listeria monocytogenes,* a safer intracellular pathogen to work with, and treated for five hours with selected compounds at various concentrations. Three *L. monocytogenes* strains were used in order to determine if the location of the bacteria within the cell had an impact on drug activity or not. The wild-type and ActA-negative strains, which escape the initial phagosome and reside in the cytosol of the macrophage, were killed within the five hour window; however, the strain that could not escape the phagosome, DP-L2319, was not significantly reduced. While investigating the intracellular efficacy of the selected compounds, it was observed that compound SK-03-92 caused morphological changes and detachment of the macrophage cells from the tissue culture plate. It appears the drug caused some form of programmed cell death, although which pathway remains to be further clarified.

ACKNOWLEDGEMENTS

I would like to first thank my major advisor, Dr. Marc Rott, for his guidance, support and wisdom throughout this project. Your patience and knowledge are what made this come together. I would also like to sincerely thank Dr. William Schwan for providing assistance and advice. I thank my other committee members, Dr. Aaron Monte and Dr. Diane Sewell, as well, for serving on my thesis committee and encouraging me through this process. I thank Dr. David Howard for helping me with the microscopy portion of my project, especially the time he took teaching me confocal techniques.

I thank the Gundersen Microbiology Research Laboratory team for assisting me and trouble-shooting with me. You didn't have to but you did and I'm very grateful for your willingness to help me. A special thanks to Dr. Charles Frevert and his lab at the University of Washington for teaching me tissue culture techniques and supplying my macrophage cell lines. Without you, none of this would have happened. Also, thanks to Dr. Daniel Portnoy at UC-Berkeley and Dr. Douglas White of Gundersen Health Systems for providing two of my *Listeria* strains. Your contribution brought depth to my project.

And most of all, I thank my family and friends for supporting me throughout this process and for believing in me the whole time. Gerald, your patience and willingness to travel to Wisconsin kept our relationship together and motivated me to push forward. Thank you for your love and encouragement.

This work would not have been possible without financial support from the UW-L Graduate Research, Service, Education and Leadership grant.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

Antibiotic resistance. Emerging antibiotic resistance is a concern that has gained international attention (1–3). Drug-resistant *Streptococcus pneumoniae* and *Campylobacter* infect over 1.5 million people in the US each year (4). According to the World Health Organization (WHO), tuberculosis is in the top 15 leading causes of death worldwide and there are about 9 million new cases annually (5–7). Over 440,000 (approximately 5%) of the new cases of tuberculosis are caused by multi-drug resistant (MDR) strains. These MDR strains cause over 150,000 deaths per year (7). Over 84 countries to date have identified at least one case of extensively-drug resistant (XDR) tuberculosis (8). Totally-drug resistant (TDR) *M. tuberculosis* is resistant to all drugs and cases have been reported in at least four countries (9). In the United States, over 70% of hospital-acquired infections (HAI) are due to bacteria that are resistant to one or more antibiotics, according to the Infectious Disease Society of America (10). Two of the most frequent causes of HAIs are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* species (11). The National Safety Healthcare Network conducted a large scale study of 463 hospitals and healthcare-associated infections during 2006-2007. They found that over 56% of pathogenic *S. aureus* strains isolated were methicillin-resistant, and over 33% of pathogenic *Enterococcus* strains were vancomycin-resistant (11).

Bacteria have acquired numerous mechanisms of drug resistance in order to survive in the presence of previously effective compounds. Efflux pumps remove the

drug from the cell as soon as it enters. Modification of the drug to a non-toxic form inactivates the drug. Altered molecular structure of drug targets may prevent drug binding. Altered membrane permeability will not allow the drug to enter the bacterial cell. Resistance mechanisms are acquired either through mutation or gene transfer (12). Bacteria are able to exchange genetic material through conjugation and the sharing of resistance plasmids (13, 14). Similarly, bacteriophages can transport genetic material from one bacterium to another through transduction (15), and bacteria can incorporate new genetic material containing resistance genes via transformation (16). Antibiotics themselves can accelerate development of resistance if they induce the SOS response in bacteria, thereby increasing the rate of mutation and the likelihood of a resistant strain emerging (17).

There are a number of ways to combat bacterial infections. Vaccination has proven highly effective, as has education and improved personal hygiene. Hand washing campaigns in hospitals have had a large impact as well (18). However, these are preventative measures that do not address how to respond to an established infection. In cases where surgical debridement or removal of the infected tissue is necessary, treatment can be severe. Other options include using probiotics or fecal transplants following *Clostridium difficile* infections to help re-establish normal flora (19, 20). Most often, physicians turn to antibiotics to inhibit bacterial growth and clear an infection.

Current pipeline. The "golden era" of antibiotics began in 1930 with the discovery of penicillin and continued for 40 years with the development of several classes of antibacterial drugs. Since then, most new drugs have been derivatives based on chemical scaffolds previously employed. From 2000-2010 there were 20 new

antibacterial drugs approved by the FDA, yet only three were of new antibiotic classes (14, 21). The other derivative-based drugs do not have a novel mechanism of action, meaning they share a binding site with drugs already on the market. Thus, the potential for emergence of drug-resistant bacterial strains is high (22).

In an effort to stem the spread of resistant bacterial infections, legislation has been passed to incentivize pharmaceutical companies to invest in antibacterial drug research (Generating Antibiotic Incentives Now Act passed July 2012) (23). Unfortunately, profit margins are lower for antibiotics than other drug therapies, and this bill may not be enough to gain the attention of big pharmaceutical companies (24). The highest selling drugs are ones that treat chronic conditions such as heart disease and high cholesterol, acid reflux, mental health, and arthritis (25). Drug development has also taken a shift towards antiviral treatments for chronic HIV and hepatitis infections that require a lifetime of treatment and thus a continuous cash flow for pharmaceutical companies (22). Similarly, with the aging United States population, the focus of pharmaceutical development has been on treatments for cancer and genetic disorders rather than acute microbial infections (25, 26). The need remains to find treatments against drug-resistant pathogens, given that infectious disease is the third leading cause of death in the United States and the second leading cause worldwide (22).

From 2011 to 2012, the FDA Center for Drug Evaluation and Research approved a number of new molecular entities, but only three are antibacterial, one of which is antimycobacterial (26, 27). As of late 2011, there were over 40 new antibacterial compounds undergoing clinical trials and a new drug application had been submitted for another antibacterial compound (21). Twelve of these compounds have a new mechanism

of action, a significant increase over recent years. The escalation in novel compounds is encouraging because an average of only 6.25% of drugs in Phase I will ever make it to market (28). The majority of these new drugs are focused on treating infections caused by drug-resistant *S. aureus*, *C. difficile*, *P. aeruginosa*, and *Acinetobacter* species. Six of the new antibacterial compounds have antimycobacterial activity, and one has proven effective against pulmonary and multi-drug resistant tuberculosis (14, 21). This new antimycobacterial drug, bedaquiline (Sirturo), was approved in 2012, making it the first novel treatment for tuberculosis to be approved in over 40 years (27).

Over one third of the world's population is latently infected with tuberculosis, yet the lack of treatments designed to combat this disease is not surprising. The cost of developing an antitubercular drug is exorbitant and requires longer clinical trials than most other types of treatment, since the patient must be followed longer to ensure that the disease does not reoccur (29). With the emergence of MDR, XDR, and TDR tuberculosis, more compounds are in the pipeline undergoing clinical trials this decade than in the previous 30 years.

Tuberculosis. The members of the *M. tuberculosis* complex (MTBC), *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium canettii,* and *Mycobacterium africanum* are the most common causes of tuberculosis in humans (30). Tuberculosis is typically a pulmonary disease that begins when MTBC bacilli are inhaled as aerosols and enter the alveolar space in the lungs (31). Alveolar macrophages then phagocytize the bacteria, and the bacteria establish themselves within the macrophage and reproduce (32). In a person with a healthy immune system, any pathogens or foreign material are initially intercepted by the innate

immune system cells: neutrophils, natural killer cells, and macrophages. Macrophages destroy pathogens by first engulfing them in a phagosome, which triggers a signaling cascade within the cell. The cell fuses a lysosome with the phagosome, bringing with it an acidic environment full of degradative enzymes and reactive oxygen and nitrogen species that will most often kill the bacterium (33). The MTBC bacteria block this pathway and prevent phagosome-lysosome fusion, while maintaining the phagosome at an early stage and a higher pH (6.4) compared to a mature phagosome (31). In this way, MTBC bacteria are able to evade the immune system, establish an infection, and cause damage to the host. If an infection is left untreated or the host is immunocompromised, the bacteria can spread to the rest of the body through the bloodstream (34).

Prior to bedaquiline in 2012, the last novel drug introduced as a direct treatment for tuberculosis was rifampicin in 1963 (35). While effective, it is not enough by itself to treat an active infection and must be taken in conjunction with other first-line drugs such as isoniazid, pyrazinamide, and/or ethambutol. The treatment regimen takes an average of nine months to two years, and there can be serious side effects to the liver, in particular, as well as other organs. Patient compliance is often a problem and treatment frequently requires direct observational therapy to ensure those who are infected follow prescription directions (35). Not following treatment guidance can lead to the emergence of drug-resistant tuberculosis. Rates of MDR, XDR, and TDR infections are increasing and pose a significant danger to society both physically and economically. The WHO recommends at least 20 months of treatment with second-line drugs for any diagnosed MDR case (36). Drugs are needed that target the bacteria in new ways and work quickly. The ideal

treatment would not allow the development of resistance and would be less toxic, thereby encouraging better patient compliance.

Drug development methods. Scientists have used a number of means to identify new drugs. The traditional method is to conduct high-throughput screening of microbial secondary metabolites, especially from soil microbes, but nothing new has been brought to market using this approach for many years. More recent methods include directed drug design based on computer modeling of potential targets, and testing of naturally-derived substances (14, 37, 38). While the rational drug design concept holds promise, very few researchers to date have been able to develop drugs that meet FDA approval. Many companies are turning back to the practice of pharmacognosy, the discovery and/or modification of naturally-derived substances.

Novel antimicrobials. Throughout history, man has looked to nature to provide medicinal treatments. Penicillin came from a mold, streptomycin and tetracycline came from soil bacteria, as did one of the newer drugs whose mechanism is still unknown—daptomycin (39). A class of compounds named "stilbenes" was first discovered in 1890, but their full potential as drugs was not investigated until only about 30 years ago. Stilbenes are naturally occurring defensive compounds produced by plants, and since their original discovery, many naturally occurring stilbenes have been identified with antibacterial activities (40).

A stilbene is two aromatic rings joined by an ethylene bridge (Fig. 1). Plant metabolic enzymes add different moieties to the basic stilbene scaffold. A good example is resveratrol, a widely-studied stilbene (tri-hydroxy substituted) found in red wine, peanuts and numerous plants. Resveratrol has shown antiaging, antiinflammatory,

antioxidant, and anticancer activity in a large number of animal and human tissue culture studies (41, 42). Resveratrol has also shown antibacterial (43) and antifungal activities (44).

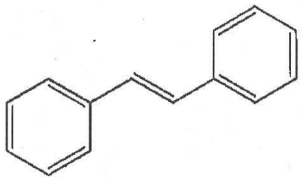


FIG 1 Simple stilbene structure

Several proprietary stilbene-derived compounds have been recently developed and investigated for antimicrobial activity (45-47). The natural product stilbene, (*E*)-3-hydroxy-5-methoxystilbene, coded CL-3, was isolated from the plant *Comptonia peregrina* (45). Compound CL-3 has a stilbene structure (Fig. 2) similar to that of resveratrol. Minimum inhibitory concentration (MIC) assays were performed on CL-3 to determine its spectrum of antibacterial activity. The compound was found to have an inhibitory effect on a number of clinically significant Gram-positive organisms, including *Bacillus anthracis* (Sterne strain), MRSA, *E. faecalis*, *Listeria monocytogenes,* and the acid-fast mycobacterial species *Mycobacterium bovis.* Building on these results, CL-3 served as a foundation for the development of a diverse array of substituted stilbenoid compounds (45–47).

Structure activity relationship (SAR) analysis of several analogs identified a phenolic stilbenoid containing a benzothiophene moiety, named SK-03-92 (45–47), with relatively low MICs against the clinically significant Gram-positive pathogens mentioned

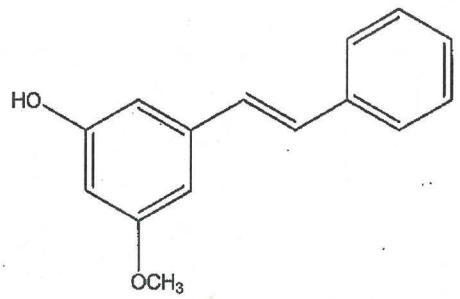


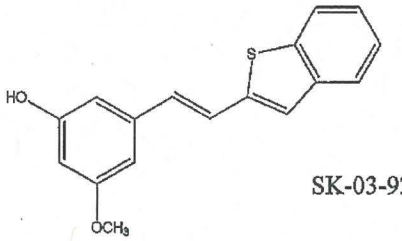
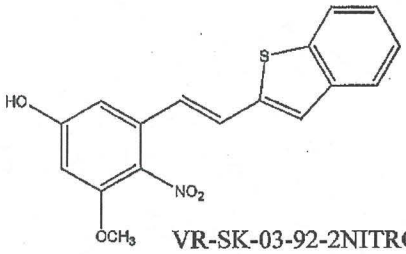
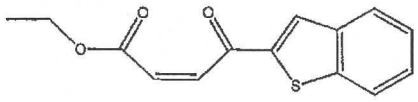
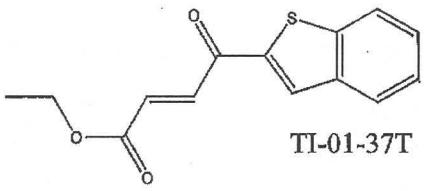
FIG 2 Parent compound CL-3

above: *S. aureus* and *E. faecalis* (Table 1)(47). SK-03-92 also showed activity against *Mycobacterium smegmatis* and *Bacillus cereus* at lower MICs than any other compounds tested to that point.

Besides SK-03-92, a number of vinyl-substituted acrylates were also synthesized, and a few of these compounds were more active against mycobacterial species than the original parent compounds (46). The low antimycobacterial MICs led to the eventual production of two acrylate compounds: TI-01-17 and its *trans*-isomer TI-01-17T.

Both acrylate compounds exhibited lower MICs against *M. smegmatis* than SK-03-92, but their MICs against clinically important Gram-positive bacteria were two- to four-fold higher than SK-03-92 (unpublished data). These SAR studies showed the benefits of the heterocyclic thiophenes against Gram-positive pathogens and the acrylate side chains against mycobacterial species. With this data in mind, the hybrid compound TI-01-37 and its *trans*-isomer were synthesized and shown to have the lowest MICs against *S. aureus* and *M. smegmatis* of the four compounds chosen for further study in this project (Table 1, unpublished data). Compound VR-SK-03-92-2NITRO, an analog of the lead compound SK-03-92 that has been modified to include a nitro group, was included in the study to better understand the mechanism of action of SK-03-92 VR-SK-03-92-2NITRO had no efficacy against a mycobacterial species but possessed activity

TABLE 1 Minimum inhibitory concentrations (MIC) of select antimicrobial compounds against Gram-positive and mycobacterial species

Compound	MIC (μg/ml)[a]			
	Staphylococcus aureus	*Enterococcus faecalis*	*Bacillus cereus*	*Mycobacterium smegmatis*
 SK-03-92	2	4	2	32
 VR-SK-03-92-2NITRO	2	ND	1	128
 TI-01-37	1	4	ND[b]	4
 TI-01-37T	1	ND	ND	ND

[a] MICs represent the average of three assays for each bacterium.
[b] Not determined

against Gram-positive pathogens (Table 1, unpublished data). Compound VR-SK-03-92-2NITRO was included as a means of perhaps. Although the compounds of interest were able to inhibit bacterial growth extracellularly *in vitro,* there is no data to show that these same compounds are able to penetrate infected human cells and kill the intracellular bacteria.

Intracellular pathogens. Select compounds from our synthetic library inhibit the growth of a wide variety of bacterial species (Table 1), including *S. aureus*, *B. anthracis*, *L. monocytogenes*, and *Mycobacterium* species (45–47). A number of these species are facultative intracellular pathogens that are able to invade host cells, enabling them to evade the human immune system.

Intracellular pathogens fall within two broad categories: those that are engulfed within a host cell and remain within the initial phagosome, and those that escape the phagosome into the cell cytosol. *Coxiella burnetii,* MTBC, and *Legionella pneumophila* are examples of bacteria that remain within the phagosome. These pathogens have means of preventing host bactericidal mechanisms from performing their function, often through the production of enzymes that break down host proteins or through the expression of genes designed to help the bacteria survive in the acidic environment within the phagosome (48). The bacterial arsenal also halts phagosomal maturation, as in the case of MTBC, or, like *C. burnetii,* produces protective measures that inhibit lysosomal breakdown of the bacteria. Other bacteria escape the phagosome and remain free in the nutrient-rich host cell cytosol. Examples of cytosolic bacteria include *Shigella flexneri, L. monocytogenes,* and *Francisella tularensis* (49). This is accomplished through the secretion of bacterial proteins that either degrade or puncture the phagosomal membrane,

releasing the bacterium into the cytosol. However they achieve their escape, these bacteria are protected from both the phagosomal defenses and the immune system defenses. The only way to combat them is to treat the host with an antimicrobial that can penetrate the outer eukaryotic cell membrane and target the bacteria where they reside.

Pharmacokinetics/pharmacodynamics of intracellular drug activity. The environment within a macrophage is complex. Numerous studies have shown that drugs that are inhibitory or bactericidal *in vitro* have little or no impact on bacteria inside a eukaryotic cell (50–54). Researchers in drug development investigate the extracellular activity and metabolism of drugs in detail, yet the intracellular effects are often not well elucidated (50). Within the cellular environment, many bacteria are able to evade host defenses and most antibiotics. Scientists conducting cellular pharmacokinetic studies concentrate on how well a drug can penetrate the target cell membrane, where it is distributed within the cell, if it is degraded by cellular enzymes, and the efflux rate of the drug out of the cell (51). There is no proven correlation between intracellular concentration and activity of a given antimicrobial (50, 51). A lack of intracellular activity could be due to a number of factors. For example, if a drug penetrates a cell but localizes at a high concentration to the lysosomal compartment, it will be inactive against bacteria such as *F. tularensis* or *S. flexneri*, which escape the phagosome and enter the cytosol (51).

Not only must the impact of the cellular environment on drug efficacy be addressed, but bacterial growth changes must be taken into account. When introduced to stressful surroundings, bacteria will express additional proteins or diminish their metabolism in order to protect themselves from the hostile environment (51). Changes of

this nature can make them more or less susceptible to antibiotics, depending on the target of the drug.

Experimental design. The purpose of this project was to determine if selected stilbene and acrylate compounds can enter an infected human macrophage cell and remain active in killing bacteria. In order to accomplish this goal, a model system was developed using an immortal human macrophage cell line that was then infected with *L. monocytogenes*.

Listeria monocytogenes, a facultative intracellular Gram-positive pathogen whose growth our compounds inhibit *in vitro*, was chosen as the model organism for a number of reasons. *Listeria monocytogenes* is a relatively safe and less virulent pathogen to work with compared to *Mycobacterium* spp. Also, *Listeria* grows much faster than *Mycobacterium* spp. (overnight versus three weeks). And as well, *L. monocytogenes* has been used extensively in the past to determine the intracellular potency of antibiotics (53--55).

Listeria monocytogenes is a rod-shaped, non-spore forming, highly motile bacterium that grows in a variety of environments. It can survive in an environment of pH 4.0-9.5, temperatures from 1°C to 45°C, and salt concentrations up to 10% NaCl (56). The bacterium is regularly found as a food contaminant, given the harsh environments it can withstand. Most often, *L. monocytogenes* enters the body through the intestinal lining when contaminated food is ingested. It can cross the placental, blood-brain, and intestinal barriers, and can cause significant disease in humans. Once inside the bloodstream, *L. monocytogenes* infects a number of cell types, including macrophage cells of the innate immune system. *L. monocytogenes* first attaches to the macrophage cell through the

interaction of bacterial internalins binding host cell surface proteins. Then, the macrophage engulfs the bacterium in a phagosome. Next, *L. monocytogenes* escapes the phagosome by secreting listeriolysin O, a pore-forming cytolysin that perforates the phagosomal membrane. By secreting two phospholipases, which further degrade the phagosome, the bacterium is released into the host cell cytosol. Wild-type (WT) *L. monocytogenes* also contains a surface-associated protein, ActA, which polymerizes host cell actin and allows movement through and between adjacent cells, leading to further spread of the bacterium throughout the body (57).

In order to test the ability of the selected novel compounds to penetrate human macrophage cells, including penetration of the outer cellular membrane and an inner phagosomal membrane, different mutant strains as well as wild-type (WT) *L. monocytogenes* were used to infect human macrophage cells (Table 2, Materials and Methods). By using these strains, it was possible to determine whether the compounds can cross both the outer cell membrane as well as the inner compartmental membrane surrounding the phagosome and remain active in killing bacteria.

Project objectives. Differentiated THP-1 human macrophage cells were infected with strains of *L. monocytogenes* and then treated with novel compounds in order to:

1. Quantify the killing effect of the selected novel compounds against intracellular *L. monocytogenes.*
2. Describe morphological changes in the macrophage cells and the bacterial cells as a result of treatment with the selected compounds.

MATERIALS AND METHODS

Cell lines, growth conditions and differentiation, and bacterial strains used in this project. THP-1 human monocyte cells, generously provided by Dr. Charles Frevert of the University of Washington, were the primary cell line used in this project (Table 2). THP-1 cells are an immortal cell line of human monocytic leukemia origin (58). THP-1 human monocyte cells were grown in RPMI 1640 medium (Cellgro, Manasses, Virginia) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific,Waltham, Massachusetts), 2 mM L-glutamine, 10 U/ml of penicillin with 10 μg/ml streptomycin (pen-strep) (MP Biomedicals, Solon, Ohio) and incubated at 37°C in 95% humidity with 5% CO_2 until confluent. The viable macrophage cell concentration was determined using the trypan blue dye exclusion method (59). The cells were differentiated from monocytic-type cells into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA, Fisher Scientific, Hampton, New Hampshire) (1 mM in RPMI 1640 medium with 1% FBS, 2 mM L-glutamine, and without pen-strep). U937 human monocyte cells (60), also provided by Dr. Charles Frevert, were used in preliminary cytotoxicity testing and were grown in the same conditions and differentiated as described above for THP-1 cells (Table 2). The bacterial strains used are described in Table 2. The WT *L. monocytogenes* strain (61) was kindly provided by Dr. William Schwan of UW-La Crosse, strain DP-L1942 (62) was generously donated by Dr. Douglas White of Gundersen Health Systems in La Crosse, and strain DP-L2319 (63) was donated by Dr. Daniel Portnoy of the University of California-Berkeley.

TABLE 2 Bacterial strains and cell lines used in this project

Bacterial strain or cell line name	Genotype	Phenotype	Source	Reference
Listeria monocytogenes EGD	WT	WT	Dr. William Schwan[a]	(61)
Listeria monocytogenes DP-L1942	*ΔactA*	Escapes phagosome, can't polymerize actin for intercellular spread	Dr. Douglas White[b]	(62)
Listeria monocytogenes DP-L2319	*Δhly, ΔplcA, ΔplcB*	Cannot escape phagosome	Dr. Daniel Portnoy[c]	(63)
THP-1 human monocyte cells			Dr. Charles Frevert[d]	(58)
U937 human monocyte cells			Dr. Charles Frevert	(60)

[a] University of Wisconsin—La Crosse, Wisconsin
[b] Gundersen Health Systems, La Crosse, Wisconsin
[c] University of California—Berkeley, California
[d] University of Washington—Seattle, Washington

Minimum inhibitory concentration assay. The Clinical and Laboratory Standards Institute guidelines (64) were used to determine the minimum inhibitory concentrations (MICs) of the compounds to be tested with the following changes: lysed horse blood was not added to the diluent cation-adjusted Mueller Hinton Broth (CAMHB). Briefly, WT, DP-L1942 (ActA-negative), and DP-L2319 (phagosomal-escape deficient) strains were grown statically overnight in tryptic soy broth (TSB) at 37°C. Aliquots were diluted in TSB to an absorbance at OD_{600nm} of 0.1 (~1 x 10^8 CFU/ml) and each culture was then diluted 1:100 in CAMHB to a final concentration of ~1 x 10^6 CFU/ml.

The compounds of interest in DMSO were diluted in CAMHB to a starting concentration of 64 μg/ml. One hundred μl of each drug was dispensed into the first well of a 96-well plate and two-fold serially diluted in CAMHB to 0.25 μg/ml. Fifty microliters of the bacterial suspension was added to each well, bringing the total volume to 100 μl, and the plate was incubated for 18-20 h at 37°C. This was repeated separately for each *L. monocytogenes* strain with all compounds. Controls included the following: a positive growth control well for each strain that contained only medium, bacteria and 1% DMSO (solvent of compound stock solution), and a negative control well containing only medium and 1% DMSO. Ampicillin was used as a positive control, as it has a published MIC for *L. monocytogenes* (64). The MIC was determined as the lowest concentration that visibly inhibited growth of the *Listeria* strains. The mean was taken of at least three separate biological assays for each compound tested against each bacterial strain.

MTT cytotoxicity assay. Cytotoxicity was determined using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay (ATCC,

Manasses, Virginia)(65). Live cells took up the yellow MTT tetrazolium salt reagent and reduced it to purple formazan crystals. As the concentration of the test compound was increased and became more toxic, fewer crystals were formed. In this way, the concentration of each compound that caused cell death in 50% of the cells was determined compared to an untreated control. Initially, 50 µl of differentiated macrophage cells were seeded into a 96-well flat bottom tissue culture-treated microtiter plate (Plate #351172, Corning Life Sciences, Durham, North Carolina) at a concentration of ~1.6 x 10^6 cells/ml, and incubated 24 h at 37°C in 5% CO_2. The medium was aspirated and 100 µl of compound was added, beginning at a concentration of 250 µg/ml (from a 10.24 mg/ml stock solution suspended in DMSO, then diluted in RPMI 1640 medium with 1% FBS, 2 mM L-glutamine, and without pen-strep). The test compound was then serially diluted across the plate down to 2 µg/ml, resulting in a total volume of 50 µl per well. Controls included DMSO (compound solvent) at 2.5% with cells, mitomycin C (positive control for immunosuppression)(66) with cells, and medium alone. Plates were incubated for 24 h at 37°C in 5% CO_2. Per the manufacturer's instructions, 10 µl MTT reagent was added per well and the plate was incubated for 2-4 h at 37°C in 5% CO_2, until purple formazan crystal formation was visible microscopically at 200X magnification. Lastly, 100 µl of detergent (10% SDS) was added per well (final concentration 6.3%), and the plate was gently swirled and incubated overnight at 37°C in 5% CO_2. The absorbance of the wells on the plate was read in a microtiter plate spectrophotometer (Molecular Devices Vmax kinetic microplate reader) at 570 nm. Assay controls were run in duplicate, and the mean of at least three biologically separate assays was averaged. Cytotoxicity (half-maximal inhibitory concentration, IC_{50}) was

determined as the concentration at which 50% of the macrophage cells were no longer metabolically active compared to the untreated control.

Intracellular penetration assay. To determine the bactericidal effectiveness of compounds inside a cell, THP-1 macrophages were infected with *L. monocytogenes*, the extracellular bacteria were removed, and the infected macrophage cells were treated with compound. The amount of live bacteria recovered from the treated macrophage cells was quantified and compared to that of an untreated control via direct plating of serial dilutions.

Briefly, THP-1 cells grown in RPMI 1640 medium with 10% FBS and pen-strep were differentiated using 1 mM PMA, then seeded in a 6-well multiplate (Plate #08-772-1B, Corning Life Sciences, Durham, North Carolina) at a concentration of ~5 x 10^5 cells/ml and allowed to incubate for 24 h at 37°C in 5% CO_2. The medium was removed and replaced with RPMI 1640 without antibiotics and with 1% FBS and incubated for 24 h at 37°C in 5% CO_2. Initially, the macrophage cells were infected with either WT, DP-L1942 (ActA-negative), or strain DP-L2319 (phagosome-escape deficient) *L. monocytogenes* at a multiplicity of infection (MOI) of 5:1, 10:1, and 20:1, bacteria to macrophage, respectively, and incubated at 37°C in 5% CO_2 for 1 h to allow phagocytosis (53). The difference in MOIs between the strains is due to the varying degree of virulence and impaired growth between the strains (62, 63). The medium was aspirated and replaced with fresh medium containing the bactericidal antimicrobial gentamicin (50 µg/ml, initially dissolved in sterile DI water) and incubated for 45 min at 37°C in 5% CO_2, to kill any non-phagocytosed bacteria (67). After the gentamicin-containing medium had been aspirated, the cells were washed four times with pre-warmed (37°C)

phosphate buffered saline (PBS) (53). Next, compound diluted in medium without antibiotics was added to the wells (at concentrations of 8 or 20 μg/ml) to determine the efficacy of intracellular penetration and to compare to the *in vitro* MIC. At T=0 and at T=1 h, the medium in the wells was aspirated and the cells were rinsed with 1 ml cold (approximately 4°C) PBS in order to wash compound from the wells. Then the cells were gently lysed using sterile DI water (2 ml per well for 10 min). At T=5 h, the same process was followed with the following alteration. The compounds were observed to cause approximately 55% of the macrophage cells to become non-adherent by 5 h. To include those cells in the data, the medium was removed from each well and pelleted for 2 min at 2500 x *g* in microcentrifuge tubes. The supernatant was removed and 1 ml cold PBS was added. The cells were again pelleted by centrifugation for 2 min at 2500 x *g* and the supernatant removed. Sterilized DI water was added (1 ml) to the cells and incubated at 10 min room temperature for lysis. Concurrently, 1 ml cold PBS was added to the adherent macrophage cells still in the 6-well multiplate, then removed and 1 ml sterile DI water was added to each well and allowed to incubate for 10 min at room temperature. At the end of the 10 min lysis incubation, the lysed non-adherent macrophages were added back to the corresponding well in the multiplate and the well contents were gently pipetted up and down in order to mix fully.

A viable bacterial cell plating assay of the total suspension was then conducted by diluting 100 μl of the specimen from the well in sterile DI water (10-fold dilutions) and plating on tryptic soy agar. The plates were incubated for 24 h at 37°C and colonies counted to quantify the number of CFUs present. Moxifloxacin (initially dissolved at 100 μg/ml in sterile DI water (68), then diluted to 4 μg/ml in each well), which has been

proven to enter macrophages and kill *L. monocytogenes* within 5 h, was used as a positive control (53). Other antibiotics have been shown to kill intracellular *L. monocytogenes*, but not within a five-hour window (69).

In subsequent experiments testing the difference in intracellular bacterial loads in the non-adherent macrophage cells in the medium versus the adherent macrophages in the wells, the procedure above was followed except the two suspensions were not combined and separate viable bacterial cell plating assays were run.

Cellular protein content. Cellular protein content of the total suspension from each well was determined using the modified-Lowry protein assay (Thermo Scientific, Waltham, Massachusetts) in order to normalize the results between assays (55, 70). Sterile DI water was used as the diluent in making the bovine serum albumin standards according to the manufacturer's instructions. A standard curve was generated, allowing determination of the protein content in the lysed macrophage cell suspensions. Bacterial survival was expressed as CFU/μg protein.

At least three separate biological experiments of both the intracellular penetration assays and the corresponding cellular protein content assays were run and the results were averaged. Statistical significance was determined using the *t*-test and was completed by the UW-La Crosse Statistical Consulting Center.

Phase/DIC microscopy. Initial seeding of the 6-well multiplates occurred as described above with the following change. Prior to adding the differentiated macrophages to each well, a single sterile coverslip was placed in the bottom of the well. The macrophage cells attached and grew on top of the coverslip, which allowed easy transfer to a slide for viewing using a microscope. Following 0, 1 h, and 5 h incubation

with the compounds, the wells were washed with ice-cold PBS and the coverslip was gently removed, and placed cell-side down on a slide. The slide was then transferred to a Nikon 80i microscope with either a 60X 1.4 N.A. objective or a 100X 1.3 N.A. objective. Phase and DIC images comparing treated with untreated macrophage cells were obtained and recorded with an ExiAqua camera and QImaging NIS Elements D software.

Confocal microscopy. In order to observe bacteria inside of the macrophages, fluorescent staining was performed prior to lysis of the infected macrophage cells (as described above for the intracellular penetration assays). Seeding of the multiplate with macrophages on top of coverslips was performed as previously stated. Following the rinse with ice-cold PBS, 2 ml PBS containing 6 µl BacLight LIVE/DEAD viability stain (Invitrogen, Carlsbad, California) were added to each well, including the control wells, and the plate was incubated at 37°C in 5% CO_2 for 15 min (71). The LIVE/DEAD stain contains two fluorescent dyes: SYTO 9, which stains nucleic acid material green, and propidium iodide, which stains nucleic acid material red. SYTO 9 is able to enter and stain double-stranded DNA in all cells, no matter if the cell membrane is intact or not, whereas propidium iodide is only able to enter cells that have died and whose cell membrane has become permeable. Live cells, both bacterial and eukaryotic, stain green while dead cells' nuclei stain orange/red. After the short incubation, the coverslips were gently removed from the wells and placed cell-side down on a slide on top of a drop of mounting medium (2% N-propyl gallate, 30% 0.1 M PBS with a pH of 9.0, and 70% glycerin). Nail polish was used to secure the coverslip in place. The cells were then viewed using a confocal microscope (Nikon C1 Plus TE2000u inverted confocal

microscope) with a Plan Apochromat 60X/1.4 N.A. objective. Images were obtained using Nikon EZ-C1 software (version 3.8).

RESULTS

Effect of novel compounds on intracellular *Listeria monocytogenes*. Within a 5 h window, the novel antimicrobials tested inhibited survival of intracellular WT and ActA-negative *L. monocytogenes* compared to untreated bacteria, but were ineffective against strain DP-L2319, the mutant that is unable to escape the phagosome.

Before investigating their ability to kill intracellularly, it was necessary to identify which compounds were effective at killing *Listeria* but were not toxic to eukaryotic cells. Only compounds meeting these requirements were chosen to test against intracellular *L. monocytogenes*. The selectivity index (SI) of each compound was determined. The SI of a compound is a measure of the effective concentration (minimum inhibitory concentration, MIC) against the bacteria, compared to the toxic concentration (half maximal inhibitory concentration, IC_{50}) in eukaryotic cells. Only compounds with an SI > 10 were used in the intracellular penetration assays.

The MIC of each compound was ascertained against the three strains of *L. monocytogenes* used in this project (for a list of the bacterial strains, see Table 2). Of the compounds tested, SK-03-92 and VR-SK-03-92-2NITRO had the lowest MICs (2 μg/ml) against WT *L. monocytogenes* (Table 3). Compound TI-01-37 at 2 μg/ml was as effective as SK-03-92 and VR-SK-03-92-2NITRO against the mutant strains, but not WT *L. monocytogenes*. TI-01-17T was the least effective (MIC ≥4 μg/ml) against all three bacterial strains tested (Table 3).

The cytotoxicity of the tested compounds in PMA-differentiated THP-1 human

TABLE 3 Minimum inhibitory concentrations of assayed compounds against *Listeria monocytogenes* strains

Compound	*Listeria monocytogenes* strain	MIC (μg/ml)	Range (μg/ml)	Repetitions
SK-03-92	EGD	2	(2)	3
	DP-L1942	2	(2)	3
	DP-L2319	2	(1-2)	5
VR-SK-03-92 2NITRO	EGD	2	(1-2)	4
	DP-L1942	2	(2)	4
	DP-L2319	2	(1-2)	4
TI-01-37	EGD	4	(2-4)	4
	DP-L1942	2	(1-2)	4
	DP-L2319	2	(1-2)	4
TI-01-37T	EGD	8	(4-8)	4
	DP-L1942	4	(4-8)	4
	DP-L2319	4	(4-8)	5
Ampicillin[a]	EGD	0.5	(<0.25-0.5)	4
	DP-L1942	0.5	(<0.25-1)	4
	DP-L2319	0.5	(0.5-1)	5

[a] Ampicillin was used as a positive control. Acceptable range is ≤ 2 μg/ml according to CLSI guidelines (64)

macrophage cells was then established to calculate the SI. Of the compounds tested. SK-03-92 and VR-SK-03-92-2NITRO were the least toxic to human macrophages (Table 4). Compound VR-SK-03-92-2NITRO had the highest IC_{50}, at 125 μg/ml, while SK-03-92 was two-fold lower at 62.5 μg/ml. The acrylate compounds TI-01-37 and TI-01-37T were the most cytotoxic of those tested at 15.7 and 31.3 μg/ml, respectively (Table 4). In previous MTT cytotoxicity assays using U937 human macrophage cells, the IC_{50} values for compounds SK-03-92 and TI-01-37 were 125 μg/ml and 31.3 μg/ml, respectively. Compounds SK-03-92 and VR-SK-03-92-2NITRO were the only compounds with an SI > 10 (Table 5) and were therefore chosen to test in the intracellular penetration assays. It should be noted that during the initial MIC testing, varied sensitivities were observed when the acrylate compounds (TI-01-37 and TI-01-37T) were tested against the different *L. monocytogenes* strains. The MICs of both compounds were two-fold lower against WT than against the mutant strains. This variability is most likely due to the attenuation of the mutants (62, 63), perhaps leaving them more susceptible to the compounds. The acrylate compounds were found to be too toxic against human macrophage cells to test intracellularly and therefore, this inconsistency was not investigated further.

When tested intracellularly, SK-03-92, at a concentration of 20 μg/ml, inhibited survival of WT *L. monocytogenes* by 75% at 5 h compared to the untreated control (2.37 x 10^4 CFU/μg protein and 9.65 x 10^4 CFU/μg protein, respectively, $p = 0.013$) (Fig. 3). SK-03-92 inhibited WT bacterial survival by 50% at the lower concentration of 8 μg/ml at 5 h (4.87 x 10^4 CFU/μg protein, $p = 0.050$). VR-SK-03-92-2NITRO at 20 μg/ml inhibited WT survival by 51% at 5 h (4.77 x 10^4 CFU/μg protein compared to the control growth of 9.65 x 10^4 CFU/μg protein, $p = 0.036$) (Fig. 3). Moxifloxacin, the positive

TABLE 4 Cytotoxicity of assayed compounds as determined using the MTT[a] cytotoxicity assay against PMA differentiated THP-1 human macrophage cells

Compound	IC_{50}[b](μg/ml)	Range
SK-03-92	62.5	(31.3-62.5)
VR-SK-03-92 2NITRO	125	(62.5-125)
TI-01-37	15.7	(7.8-15.6)
TI-01-37T	31.3	(31.3)

[a] 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide).

[b] IC_{50} refers to the concentration at which THP-1 cell viability was ≤ 50%.

TABLE 5 Selectivity index of assayed compounds

Compound	*Listeria monocytogenes* strain	Genotype	MIC (μg/ml)	IC_{50}[a] (μg/ml)	Selectivity Index (IC_{50}/MIC)
SK-03-92	EGD	WT	2	62.5	31.3
	DP-L1942	*ΔactA*	2		31.3
	DP-L2319	*Δhly,ΔplcA,ΔplcB*	2		31.3
VR-SK-03-92-2NITRO	EGD	WT	2	125	62.5
	DP-L1942	*ΔactA*	2		62.5
	DP-L2319	*Δhly,ΔplcA,ΔplcB*	2		62.5
TI-01-37	EGD	WT	4	15.7	3.9
	DP-L1942	*ΔactA*	2		7.9
	DP-L2319	*Δhly,ΔplcA,ΔplcB*	2		7.9
TI-01-37T	EGD	WT	8	31.3	3.9
	DP-L1942	*ΔactA*	4		7.8
	DP-L2319	*Δhly,ΔplcA,ΔplcB*	4		7.8

[a] IC_{50} refers to the concentration at which THP-1 cell viability was = 50%.

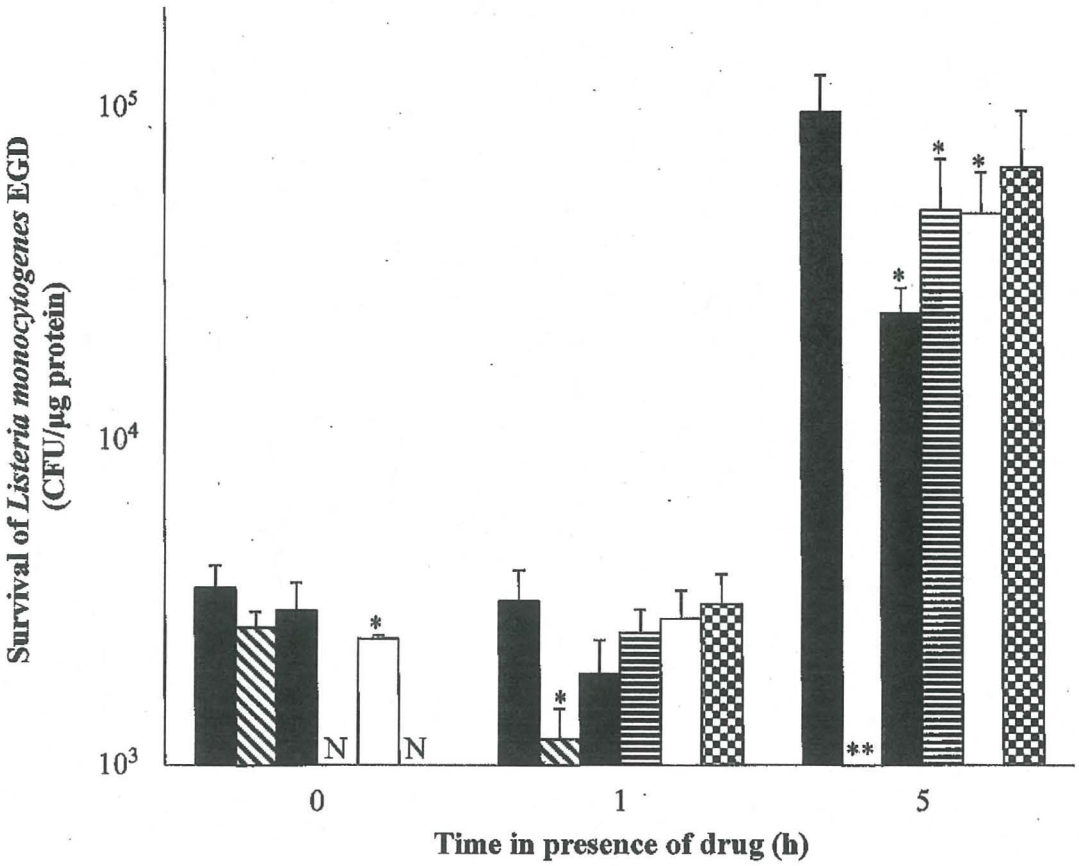


FIG 3 Intracellular survival of WT *Listeria monocytogenes* within THP-1 human macrophage cells with or without compound added. T= (0, 1, 5) refers to time in the presence of compound, after the bacteria have been allowed 1 h for phagocytosis and with the extracellular bacteria removed. Bars represent untreated cells (black) and cells treated with moxifloxacin at 4 µg/ml (diagonal stripe), SK-03-92 at 20 µg/ml (gray), SK-03-92 at 8 µg/ml (horizontal stripe), VR-SK-03-92-2NITRO at 20 µg/ml (white), or VR-SK-03-92-2NITRO at 8 µg/ml (checker). *, p value <0.05, as determined by the t-test. **, moxifloxacin at 5 h time point not shown on this scale. N, not determined. Averages of at least three separate biological assays are shown.

control, inhibited growth by >99% compared to the untreated control (36.0 CFU/μg protein and 9.65 x 10^4 CFU/μg protein, respectively, $p = 0.007$). Against the ActA-negative mutant, both compounds were reduced bacterial counts at 8 and 20 μg/ml at the 5 h time point compared to the untreated control (SK-03-92 at 20 μg/ml, 4.47 x 10^4 CFU/μg protein, $p = 0.012$; SK-03-92 at 8 μg/ml, 1.2 x 10^5 CFU/μg protein, $p = 0.029$; NITRO at 20 μg/ml, 1.07 x 10^5 CFU/μg protein, $p = 0.020$; NITRO at 8 μg/ml, 1.85 x 10^5 CFU/μg protein, $p = 0.010$; compared to the untreated cells, 2.53 x 10^5 CFU/μg protein) (Fig. 4). Only SK-03-92 at 20 μg/ml and the positive control significantly inhibited survival of the ActA-negative mutant (56% reduction by both compared to the untreated cells) at the 1 h time point (SK-03-92 at 20 μg/ml, 2.76 x 10^3 CFU/μg protein, $p = 0.025$; moxifloxacin at 4 μg/ml, 2.70 x 10^3 CFU/μg protein, $p = 0.029$; untreated cells, 6.20 x 10^3 CFU/μg protein) (Fig. 4). Neither compound at any concentration nor time of treatment had a significant killing effect against bacteria of strain DP-L2319 (phagosomal-escape deficient) (Fig. 5).

When normalizing the bacterial survival counts, it was observed that the protein concentration of the lysed macrophage/bacterial suspension increased at the 1 h time point and was substantially higher in the cells treated with SK-03-92 (both 8 and 20 μg/ml) as compared to the untreated control when infected with WT *L. monocytogenes* (Fig. 6). Compound VR-SK-02-92-2NITRO had a less pronounced effect at either concentration than SK-03-92. By 5 h, all protein content of both treated and untreated macrophage cells returned to levels similar to that at the 0 h. The protein content in the macrophages infected with the ActA-negative mutant followed a pattern very similar to that of the WT infected cells (Fig. 7). The increase was not as pronounced when the

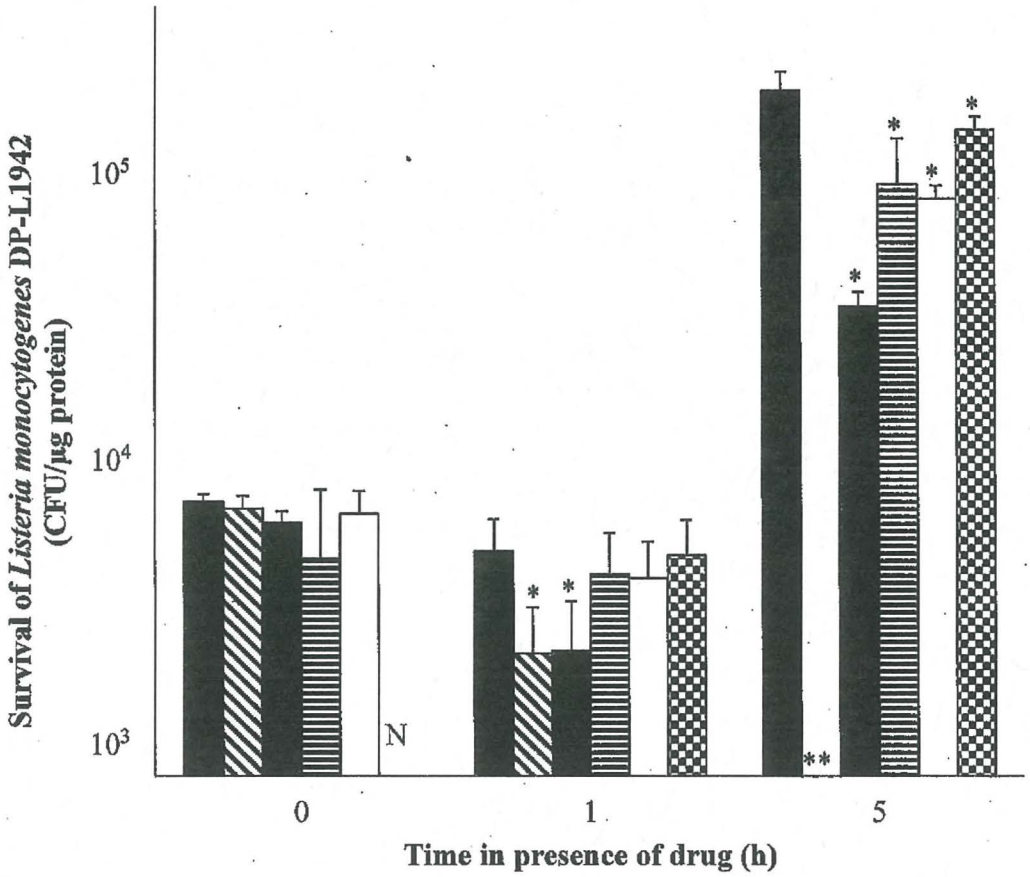


FIG 4 Intracellular survival of *Listeria monocytogenes* DP-L1942 (ActA-negative) within THP-1 human macrophage cells with or without compound added. T= (0, 1, 5) refers to time in the presence of compound, after the bacteria have been allowed 1 h for phagocytosis and with the extracellular bacteria removed. Bars represent untreated cells (black) and cells treated with moxifloxacin at 4 µg/ml (diagonal stripe), SK-03-92 at 20 µg/ml (gray), SK-03-92 at 8 µg/ml (horizontal stripe), VR-SK-03-92-2NITRO at 20 µg/ml (white), or VR-SK-03-92-2NITRO at 8 µg/ml (checker). *, p value <0.05, as determined by the t-test. **, moxifloxacin at 5 h time point not shown on this scale. N, not determined. Averages of at least three separate biological assays are shown.

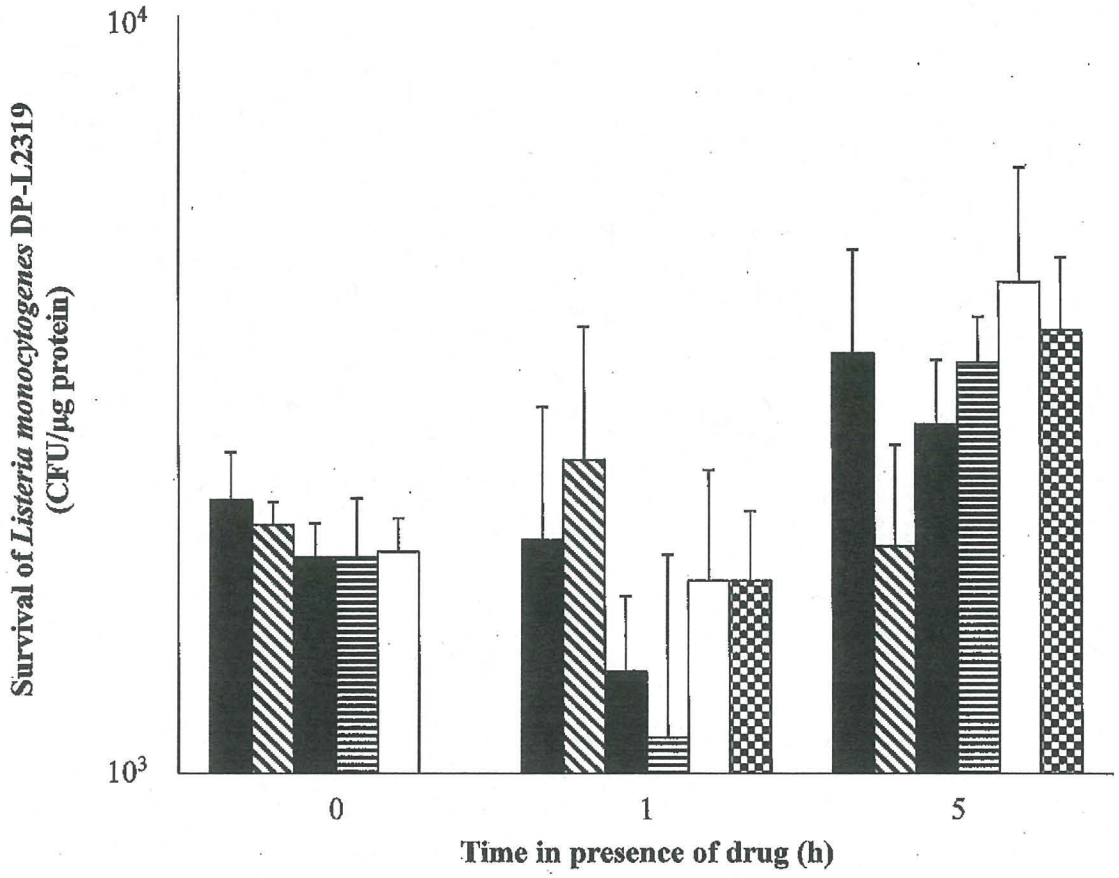


FIG 5 Intracellular survival of *Listeria monocytogenes* DP-L2319 within THP-1 human macrophage cells with or without compound added. Strain DP-L2319 is not able to escape the initial phagosome. T= (0, 1, 5) refers to time in the presence of compound, after the bacteria have been allowed 1 h for phagocytosis and with the extracellular bacteria removed. Bars represent untreated cells (black) and cells treated with moxifloxacin at 4 µg/ml (diagonal stripe), SK-03-92 at 20 µg/ml (gray), SK-03-92 at 8 µg/ml (horizontal stripe), VR-SK-03-92-2NITRO at 20 µg/ml (white), or VR-SK-03-92-2NITRO at 8 µg/ml (checker). *, p value <0.05, as determined by the t-test. N, not determined. Averages of at least three separate biological assays are shown.

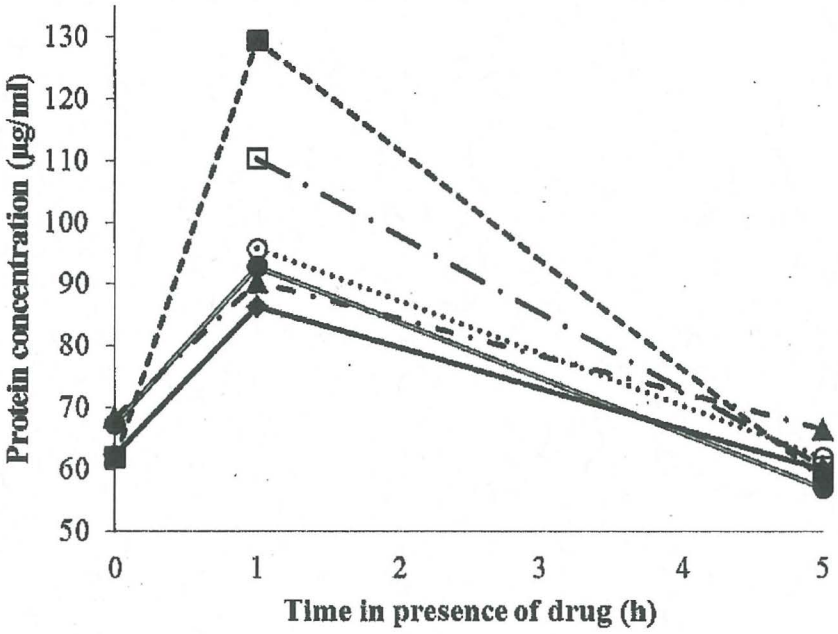


FIG 6 Protein concentration of the lysed macrophage cell suspension following infection with WT *Listeria monocytogenes.* Symbols represent untreated cells (-♦-) and cells treated with SK-03-92 at 20 µg/ml (- -■- -), SK-03-92 at 8 µg/ml (-- ·□--·), VR-SK-03-92-2NITRO at 20 µg/ml (=●=), VR-SK-03-92-2NITRO at 8 µg/ml (··○··), or moxifloxacin, the positive control, at 4 µg/ml (·-· ▲ ·-·). Data shown are representative of three separate biological assays.

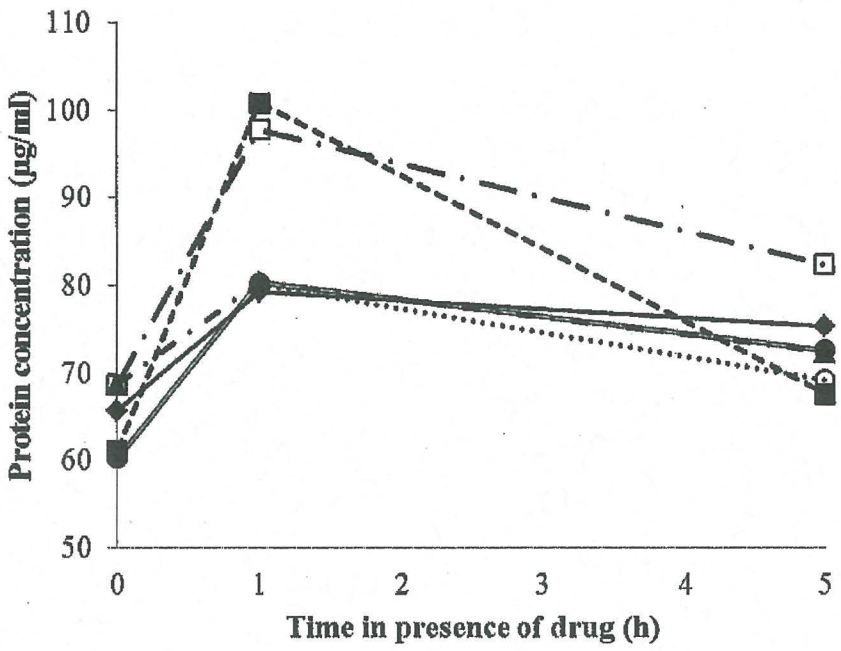


FIG 7 Protein concentration of the lysed macrophage cell suspension following infection with *Listeria monocytogenes* DP-L1942 (ActA-negative). Symbols represent untreated cells (-♦-) and cells treated with SK-03-92 at 20 µg/ml (- -■- -), SK-03-92 at 8 µg/ml (-- ·□--·), VR-SK-03-92-2NITRO at 20 µg/ml (=●=), VR-SK-03-92 at 8 µg/ml (··○··), or moxifloxacin, the positive control, at 4 µg/ml (·-· ▲ ·-·). Data shown are representative of three separate biological assays.

macrophage cells were infected with strain DP-L2319, the mutant unable to escape the phagosome (Fig. 8). However, an increase in protein content was measured at the 1 h time point and by 5 h levels had returned to values similar to the 0 h.

Effects of 20 μg/ml of compound SK-03-92 on macrophage adherence and morphology. During the initial experiments, it was observed that PMA-differentiated THP-1 macrophage cells, initially seeded at a concentration of ~5 x 10^5 cells/ml, detached from the bottom of the tissue culture multiplate wells after approximately 1.5 h in the presence of 20 μg/ml SK-03-92. Further experiments were completed to explore this phenomenon to determine what effect initial macrophage cell concentration, SK-03-92 concentration, or time in the presence of the compound had on the cell adherence.

Macrophage cells did not detach when seeded at a higher concentration (approximately 2 x 10^6 cells/ml) yet there was still a loss of viability in the remaining adherent cells (Table 6). Due to time constraints, this experiment was run only once in duplicate. The data, however, supports observations of detachment made by others (personal communication, Rachel Minerath) when working with SK-03-92. The data also explain why this effect was not noted at similar concentrations of drug when running the cytotoxicity assays, as the 96-well plates were seeded with macrophage cells at a higher concentration, approximately 1.6 x 10^6 cells/ml (see Materials and Methods).

Experiments were run to determine at what concentration SK-03-92 caused macrophage cells to detach from the plate well. Initially, macrophages were treated for 5 h with concentrations of 5, 10, 20 or 50 μg/ml of SK-03-92. It was observed that detachment was visible in cells treated with 10 μg/ml or higher of the compound. Those cells treated with 50 μg/ml appeared to clump, there was lots of debris visible, and the

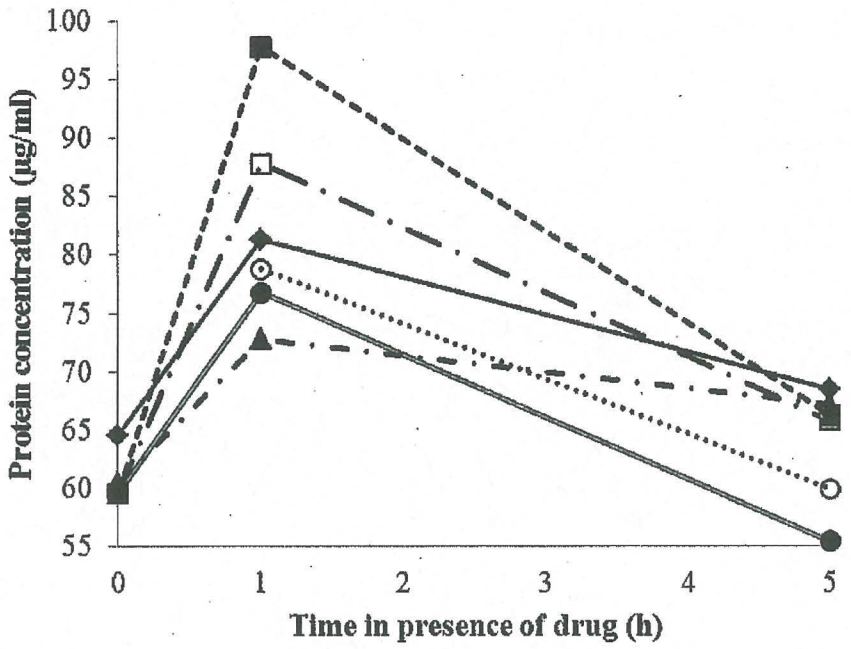


FIG 8 Protein content of the lysed macrophage cell suspension following infection with *Listeria monocytogenes* DP-L2319, which cannot escape the initial phagosome. Symbols represent untreated cells (-♦-) and cells treated with SK-03-92 at 20 µg/ml (- -■- -), SK-03-92 at 8 µg/ml (-- ·□-·), VR-SK-03-92-2NITRO at 20 µg/ml (=●=), VR-SK-03-92 at 8 µg/ml (··○··), or moxifloxacin, the positive control, at 4 µg/ml (·-· ▲ ·-·). Data shown are representative of three separate biological assays.

TABLE 6 Effect of macrophage cell concentration on macrophage cell adherence after 5 h in the presence of 20 μg/ml SK-03-92

	Total concentration (cells/ml x 10^5)	Non-adherent cells (%)	Adherent cells (%)
Untreated cells	3.7	0	100
	23.1	1	99
SK-03-92 (20 μg/ml)	2.8	73	27
	20.6	1	99

cells looked unhealthy. A single experiment was then run ranging from 0 to 10 μg/ml, measured using trypan blue dye exclusion, which indicated the shift from attached to detached occurred between 7 and 8 μg/ml of SK-03-92 (data not shown), thereby showing a concentration-dependent effect of the compound on macrophage cells as well.

When seeded at approximately 5 x 10^5 cells/ml, the macrophage cell population shifted from adherent (100% at 0 h) to non-adherent (55% at 5 h) when in the presence of 20 μg/ml SK-03-92 (Fig. 9). The increase in non-adherent cells was linear beginning at the 1 h time point. The protein concentration of the non-adherent cells in the medium and the adherent macrophage cell population followed a similar pattern when treated with 20 μg/ml SK-03-92 (Fig. 10, closed squares). In untreated macrophages, the percent adherent (99%) and the protein concentration of the populations (~5 μg/ml in the medium and ~100 μg/ml in the adherent cells) remained relatively constant (Fig. 9--10).

Survival of intracellular bacteria in the two macrophage cell populations was also compared. Macrophage cells were infected with WT *L. monocytogenes* and the non-adherent and the adherent populations were separated and lysed at specific time points. The suspensions were plated to compare growth after 1 and 5 h in the presence of SK-03-92 at 20 μg/ml (Table 7). By normalizing the results to the protein concentration, it was seen that compound SK-03-92 at 20 μg/ml inhibited bacterial survival in the adherent macrophage cells, but did not alter the bacterial survival within the non-adherent cells compared to the untreated cells (Table 7).

In order to better understand what morphological changes were occurring in the macrophage cells due to treatment with 20 μg/ml of the compound SK-03-92, microscopic images were taken comparing treated and untreated macrophages at 0, 1, and

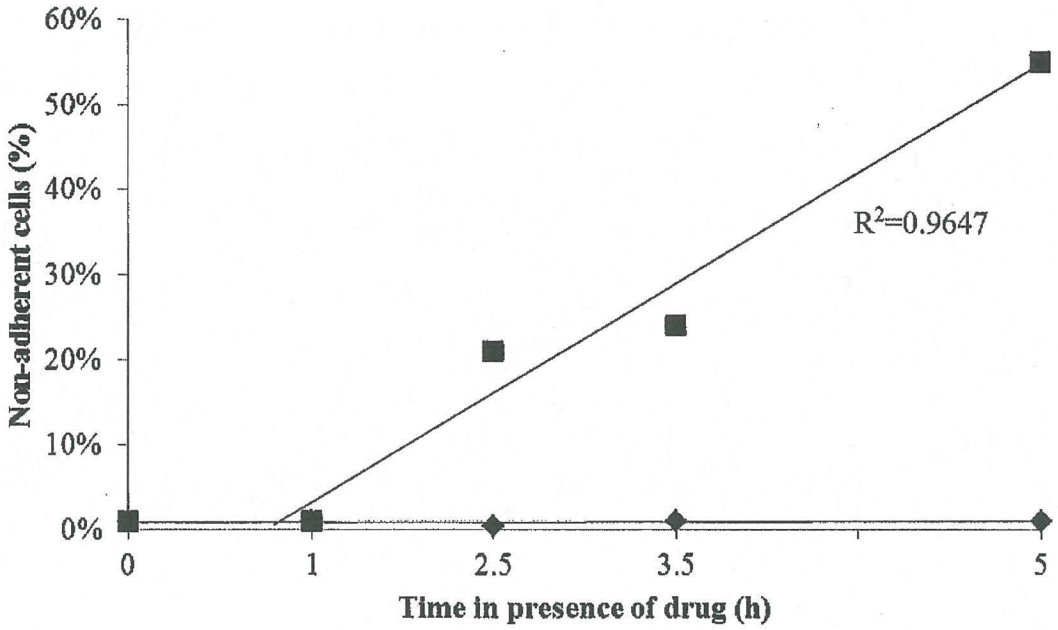


FIG 9 Change in THP-1 macrophage cell adherence over time after treatment with 20 μg/ml SK-03-92. Macrophages seeded at an initial concentration of ~5 x 10^5 cells/ml. Symbols represent the untreated cells (♦) and SK-03-92 (20 μg/ml) treated cells (■). Data are the results of one assay.

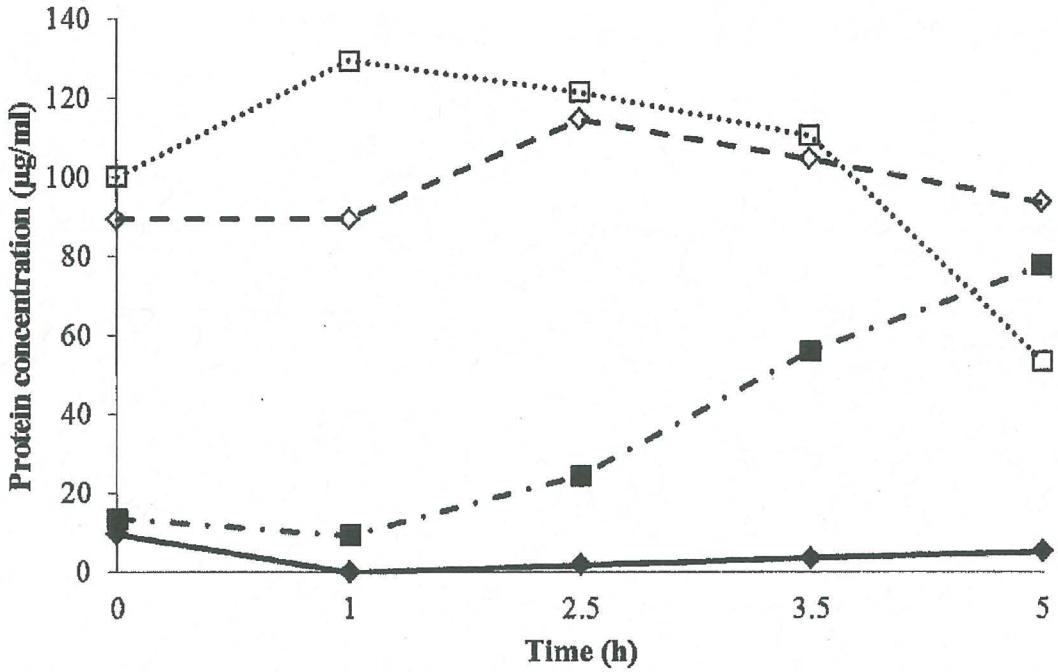


FIG 10 Protein concentration of the non-adherent and adherent THP-1 macrophage cells during the 5 h time course with and without compound added. The symbols are represented as follows: Symbols represent the untreated non-adherent macrophage cells (includes protein content from the medium) (-♦-), untreated adherent macrophage cells (- -◊- -), SK-03-92 (20 µg/ml) treated non-adherent macrophage cells (includes protein content from the medium) (·-·■·-·), and SK-03-92 (20 µg/ml) treated adherent macrophage cells (··□··). Data are the results of one assay.

TABLE 7 Survival of WT Listeria monocytogenes in the adherent and non-adherent cell populations of macrophage cells treated with 20 μg/ml SK-03-92 compared to untreated cells

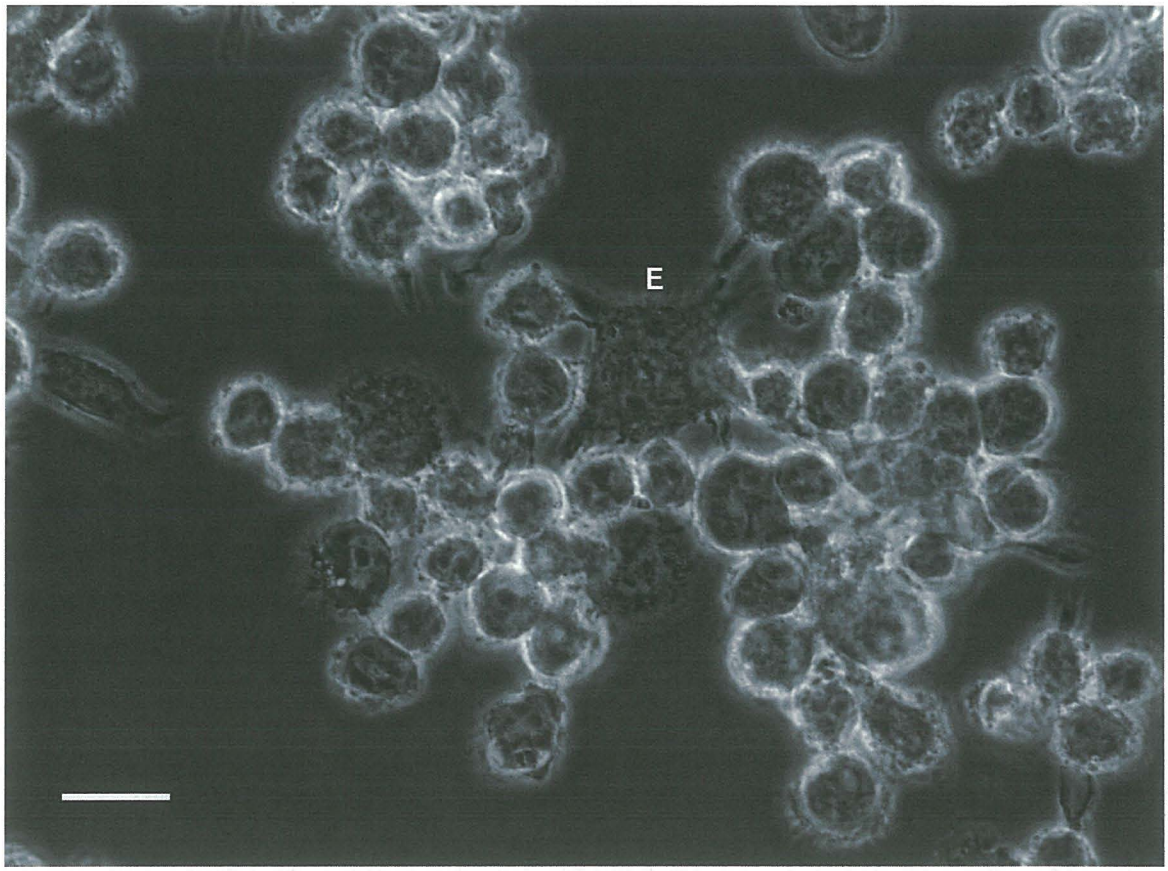
	Bacterial survival in non-adherent cells (CFU/μg protein x 10^3)	Bacterial survival in adherent cells (CFU/μg protein x 10^3)
Untreated cells[a]		
1 h	0.38	2.4
5 h	3.3	47.9
SK-03-92 treated cells[a]		
1 h	0.59	2.1
5 h	3	6.5

[a] Initial seeding of macrophage cells was ~5 x 10^5 cells/ml.

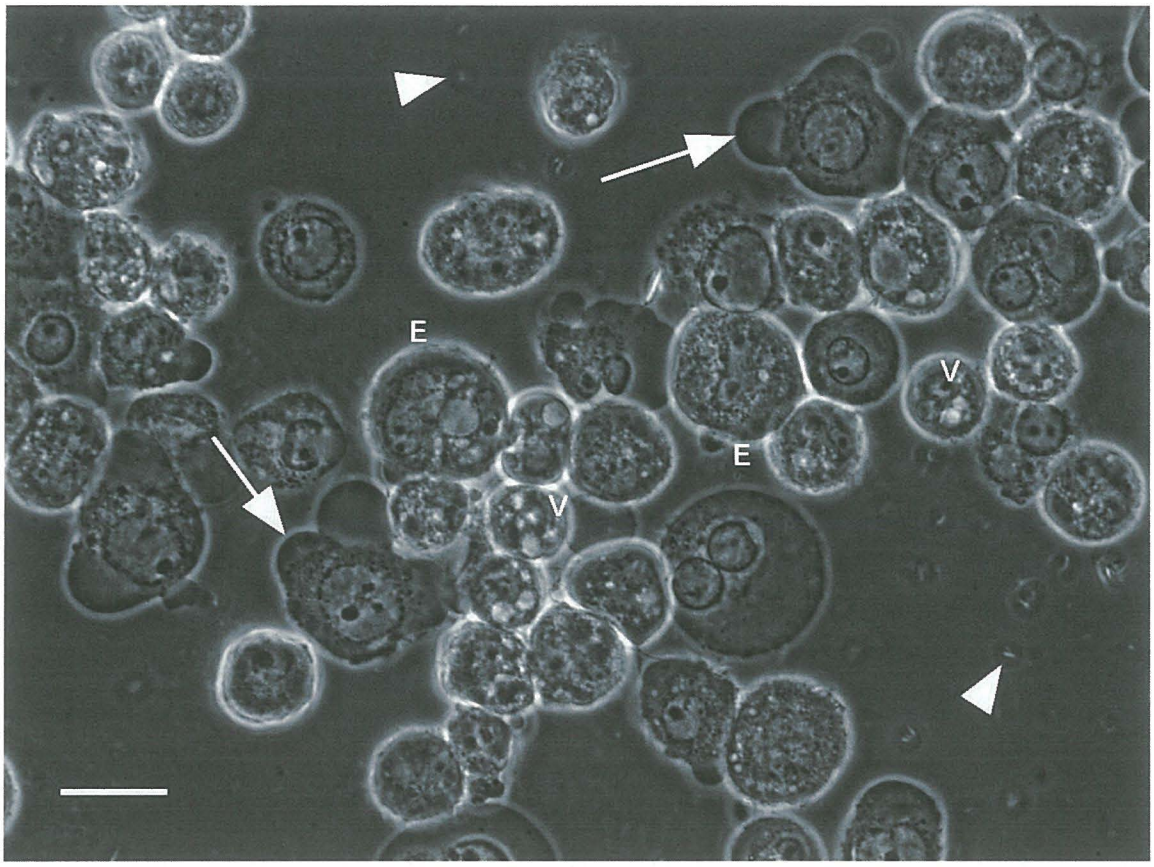
5 h post-treatment. The macrophage cells were not infected with *L. monocytogenes* so that the effects of the drug could be observed apart from the effects of the bacteria. Both treated and untreated cells appeared nearly identical at 0 h and 1 h. By five hours, there were a number of visible differences between the treated and untreated cells (Fig. 11-12). In phase contrast, gross differences were visible (Fig. 11). The untreated cells showed dendritic-like processes, which appeared to contribute to the cells' adherence to the bottom of the culture plate well. The dendritic-like processes were not visible in the treated cells at 5 h post-treatment and the cells appeared rounded and more refractile than the untreated cells. Also, the treated cell nuclei appeared larger than the untreated nuclei, and dark spots were visible inside the treated nuclei. A few of the treated cells were blebbing and a large amount of debris was visible in the surrounding medium that was not seen in the untreated cell images. When viewed at 100X in DIC, other significant disparities were clear. A number of the treated cells appeared enlarged (hypertrophy), vacuoles were visible inside the cells, and the debris that was seen in phase contrast is still evident outside of the treated macrophages in the medium (Fig. 12).

It should be noted, the morphological changes seen in the macrophage cells treated with SK-03-92 were not present in the cells treated with VR-SK-03-92-2NITRO. Some detachment of cells was noted, but there was very little debris in the surrounding medium and the macrophages visually appeared healthy (data not shown). Because the effect of 20 μg/ml of SK-03-92 on the macrophages was so strong, only those conditions were pursued for further information.

Confocal images were obtained of macrophages infected with WT *L. monocytogenes* and treated with 20 μg/ml SK-03-92 up to 5 h to look for differences in

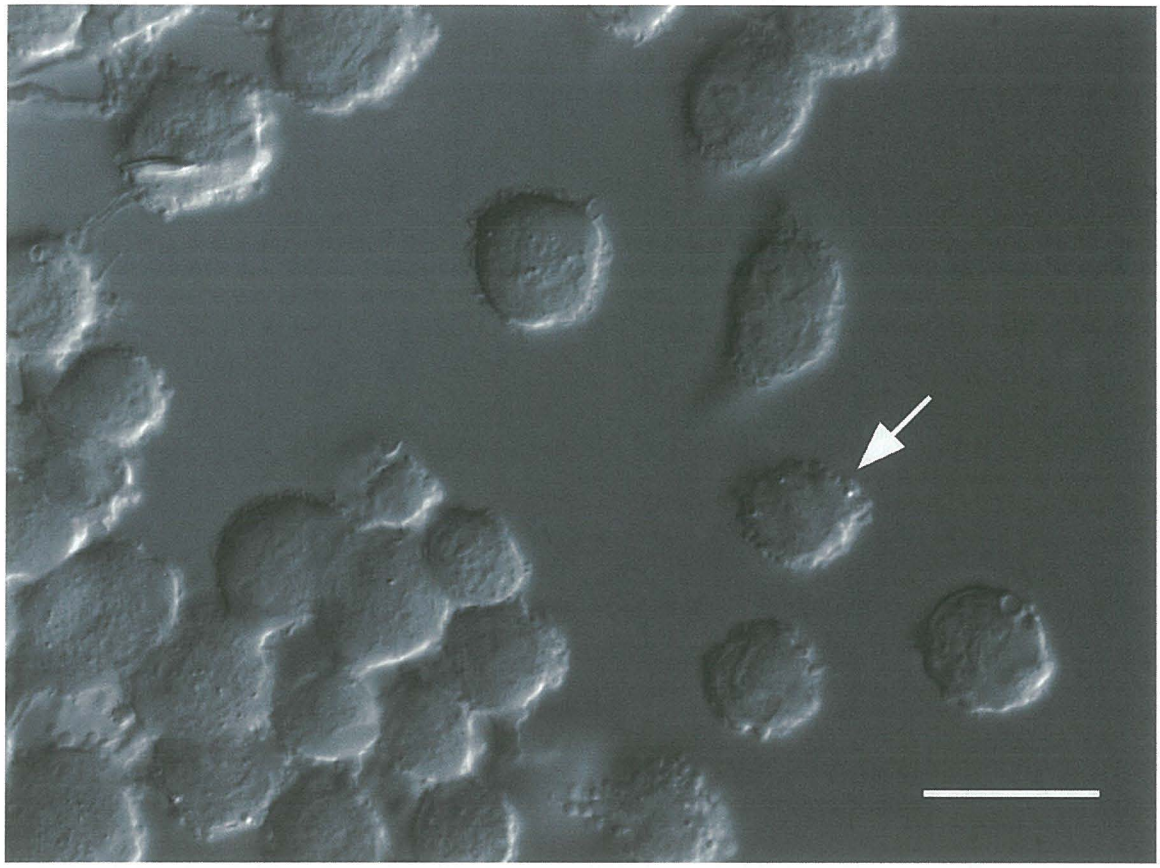


A.

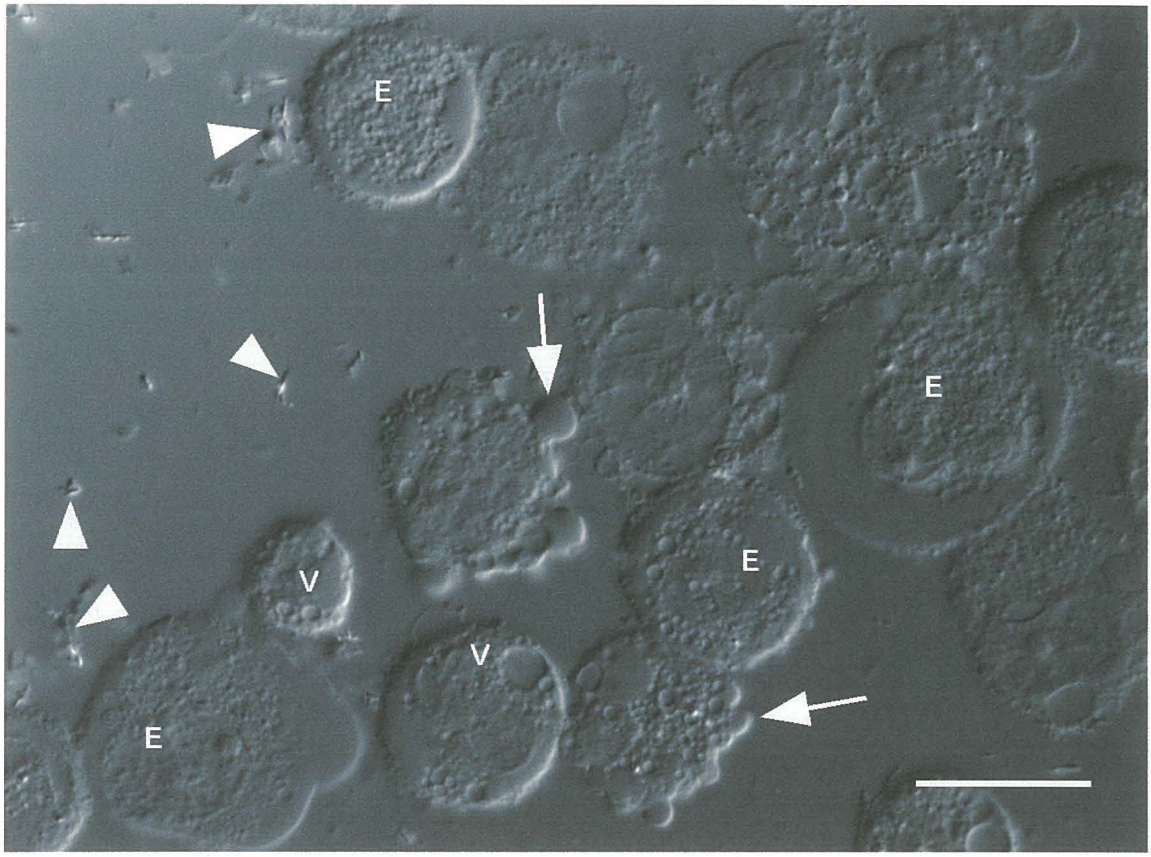


B.

FIG 11 Representative phase contrast images of untreated and SK-03-92 (20 μg/ml) treated macrophages at 5 h post-treatment, 600X under oil. Cells were not infected with *Listeria monocytogenes.* A. Untreated cells. B. SK-03-92 treated cells. Arrow = blebbing, arrowhead = debris, E = enlarged cell, V= vacuoles visible. Scale bar = 20 μm.



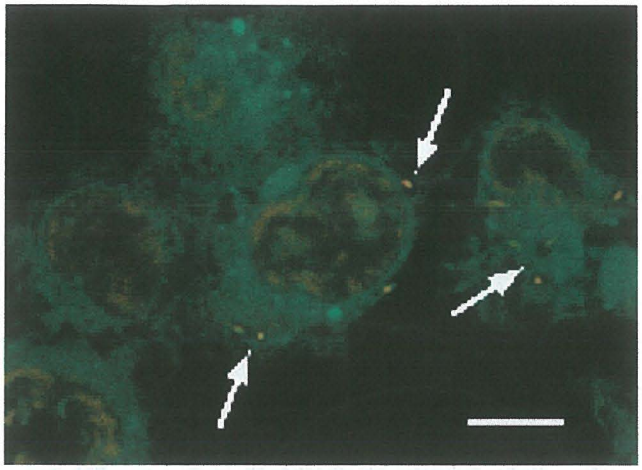
A.



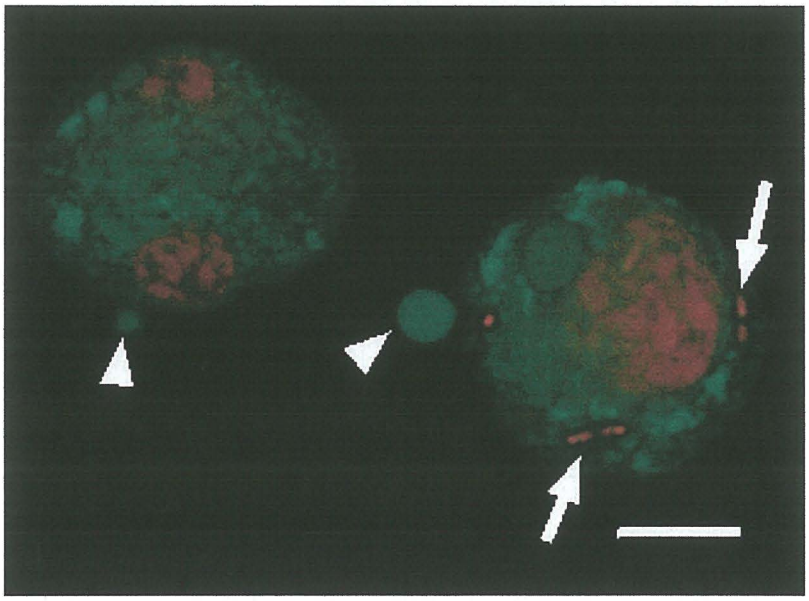
B.

FIG 12 Representative DIC images of untreated and SK-03-92 (20 μg/ml) treated macrophages at 5 h post-treatment, 1000X under oil. Cells were not infected with *Listeria monocytogenes.* A. Untreated cells. B. SK-03-92 treated cells. Arrow = blebbing, arrowhead = debris, E = enlarged cell, V= vacuoles visible. Scale bar = 20 μm.

bacterial cell morphology between treated and untreated intracellular bacteria (Fig. 13). At 5 h post-treatment, there were notably fewer bacteria present in the SK-03-92 treated macrophages than in the untreated macrophages. There was no visible difference in the bacterial morphology at the magnification used (600X oil); however, differences were visible between the treated and untreated macrophage cells. The treated macrophage cells presented blebbing and the formation of vacuoles, similar to what was noted in the phase and DIC images at 5 h. The nuclei of the treated macrophage cells appear swollen and have taken up a significant amount of the propidium iodide. Only a cell whose membrane has become permeable would take up the propidium iodide, which suggests that membrane integrity had been lost in the treated cells pictured (Fig. 13).



A.



B.

FIG 13 Representative confocal images of macrophage cells infected with WT *Listeria monocytogenes* at 5 h post-treatment with 20 μg/ml SK-03-92. Macrophages were stained with SYTO9 (green) and propidium iodide (red). Only cells whose membranes have become permeable will take up the propidium iodide. A. Untreated cells, 600X oil. B. SK-03-92 treated cells, 600X oil. Arrowhead = blebbing. Arrow = visible WT *L. monocytogenes*. Scale bar = 10 μm.

DISCUSSION

Selected compounds that had exhibited antimicrobial activity *in vitro* were tested for intracellular activity using human macrophage cells infected with different strains of *L. monocytogenes.* SK-03-92 and VR-SK-03-92-2NITRO inhibited bacterial growth inside the cytosol of the macrophage, compared to the untreated control, but were not effective against bacteria that were trapped in the phagosome. During the course of the intracellular assays, it was observed that SK-03-92 caused morphological changes and detachment of the macrophage cells in a time and concentration-dependent manner. SK-03-92 also caused an increase in protein content of treated cells at the 1 h time point compared to untreated control cells.

The tested compounds penetrated human macrophage cells and reduced survival of both intracellular WT and ActA-negative *L. monocytogenes* within 5 h compared to the untreated control. The only difference between these two strains of *L. monocytogenes* is the presence or absence of a fully functional ActA protein. The results of this study indicate that ActA is not the target of the compounds, nor does the ability of the bacteria to spread cell-to-cell impact the capability of the compound to kill. VR-SK-03-92-2NITRO was not as effective intracellularly within the 5 h test window as SK-03-92, suggesting that the addition of a nitro group decreased efficacy against intracellular *L. monocytogenes.* The MICs of VR-SK-03-92-2NITRO against the three *L. monocytogenes* strains *in vitro* were the same as SK-03-92. It is possible the nitrite group affected uptake into the macrophage cell or that it altered the way the drug was metabolized. As well, the morphological changes noted in the macrophage cells when treated with 20 μg/ml of SK-

03-92 were not seen in the cells treated with the same concentration of the nitro compound. The presence of the nitro group changed how the macrophage cells and the bacterial cells responded.

While the compounds tested decreased bacterial survival at 5 h compared to the untreated control, the bacterial counts (CFU/μg protein) increased between the 1 and 5 h time points. The bacteria appear to have multiplied during this period. The intracellular doubling time of *L. monocytogenes* is approximately 42 min (72, 53), which would correspond to an increase of over one log within four hours, as was seen in this project.

There are a number of possible explanations for the increase in intracellular bacteria recorded in the treated cells in this study. As shown in the macrophage detachment experiments, the bacterial growth observed at 5 h could be a concentration-dependent effect of the compound. If the compound entered the macrophage and bound to both macrophage and bacterial targets, or was metabolized/altered/effluxed by either the macrophages or the bacteria, there may have been insufficient drug concentration to kill all intracellular bacteria. A percentage may have been killed but survivors were able to continue growing. The growth observed in the experiments of this study is similar to that seen in postantibiotic effect studies (73) and may be a dosing issue.

Another possible contributing factor is the decrease in protein content seen from the 1 h to the 5 h time point. The decrease suggests that proteins were lost during the experiments, most likely during the wash steps following 5 h treatment with the tested compounds. The medium was removed from the wells and the non-adherent cells were pelleted by centrifugation in order to capture the loose cells. If a fraction of the cells had lysed and released their intracellular contents, the proteins would not have pelleted during

centrifugation but any bacteria released would have been. Significantly lower protein content would skew the data to show more bacterial per μg protein.

Alternately, there may be a subpopulation of bacteria that avoid killing by the novel concept of "bet-hedging," where different genes are expressed in a subpopulation of cells that permits survival of the species during stressful events, such as antibiotic treatment (74-76). The surviving cells, called "persisters," are typically slowed metabolically and are in a dormant state. Upon antibiotic treatment, most cells may die but the persisters survive and eventually reseed the population (74, 75). These persister cells don't contain any heritable traits as their progeny again become susceptible to the drugs. Bacterial persisters inside the macrophage cells may have survived treatment with our compounds.

It is feasible the window of the assay was too short to allow the compound to fully penetration the macrophages. However, this is unlikely given that the majority of the macrophages were visibly affected by five hours, meaning the drug was able to penetrate the macrophage cellular membrane.

It is likely the increase in bacteria per μg protein is a result of a combination of two or more of the scenarios outlined above. Lowered protein content multiplied with bacterial growth could yield the results that I saw with my data.

Neither drug tested was effective within 5 h against *L. monocytogenes* strain DP-L2319, which cannot escape the phagosome. These results could be due to drug inactivation before or after transport into the phagosome or a lack of penetration through the phagosomal membrane. The phagosomal membrane has been described as a double membrane formed through invagination and inversion of the cytoplasmic membrane.

The phagosomal membrane often contains markers from other organelles, such as the ER or the Golgi apparatus (77-79). It is possible that the formation of the phagosome inverts a transporter or pore, which could render transport mechanisms non-functional, thereby preventing uptake of the compounds into the phagosomal space where the bacterial cells were present.

The observation that the tested compounds do not cross the phagosomal membrane and remain active is contrary to previous data from the National Institute of Allergy and Infectious Diseases (NIAID) showing compound SK-03-92 was effective against intracellular *Mycobacterium tuberculosis* (Appendix A). The NIAID performed intracellular assays in a manner similar to the methods used in this project, with a few differences. They used J774 murine macrophages at a density of 2.0×10^5 cells/ml in 12-well tissue culture plates. The NIAID found that SK-03-92 was cytotoxic at 50 μg/ml and that it was effective (>2 log reduction of bacterial survival) at killing intracellular *M. tuberculosis* at all three concentrations tested (0.5, 5, and 50 μg/ml). The assay ran for seven days, however, before the macrophage cells were lysed and the bacterial counts compared. The long assay window is a significant difference from the 5 h window used in this study.

Members of the MTBC have long been thought to remain in the phagosome (31), and yet the results of this study suggest the compounds may not cross the phagosomal membrane. New reports propose *M. tuberculosis* escapes the phagosome after a few days and then resides in the cytosol (80, 81). The data from this project support this hypothesis given that after seven days SK-03-92 inhibited *M. tuberculosis* survival in the NIAID assays (Appendix A), yet within the 5 h window of this project, the same compound did

not inhibit *L. monocytogenes* strain DP-L2319, which cannot escape the phagosome. The timespan of the intracellular assays for this study was determined based on the attenuation of strain DP-L2319 (63). The mutant bacteria with attenuated virulence do not thrive and will die from cellular defenses after 5 h. The short window in this project is a limitation to fully understanding the differences in data regarding possible phagosomal penetration.

When testing SK-03-92 intracellularly, it was observed that the compound caused morphological changes and detachment of human macrophage cells, particularly at 20 μg/ml. The detachment of the macrophage cells was visible microscopically after 1 h and increased in a time-dependent manner up to 5 h when the cells were treated with SK-03-92. As presented in the Results section, bacteria were susceptible to SK-03-92 primarily in the adherent population at 5 h. These data suggest the detached macrophage cells are changed in some way so that the bacteria within the macrophage cells were no longer affected by SK-03-92, possibly by metabolizing or inactivating the drug in some way, or altering the macrophages' ability to take up the drug.

Infection with *L. monocytogenes* did not affect macrophage cell appearance over the time course tested. Morphological changes (blebbing, vacuoles, hypertrophy, nuclear condensation, debris in the medium) observed in the macrophage cells were visible in the cells 1 h post-treatment with SK-03-92 at concentrations > 8 μg/ml but not in untreated cells, with or without bacteria present. The presence of blebbing and vacuoles are consistent with descriptions of macrophage cells undergoing programmed cell death (PCD)(82). Programmed cell death describes the phenomenon whereby cells direct their own destruction via complex signaling pathways (83). Intracellular bacterial infection can

induce PCD in eukaryotic cells (84-86), yet visible evidence of blebbing and vacuolization were clear in treated cells that were not infected with *L. monocytogenes* in this study (Fig. 11-12). Overall, these observations point to the drug being the cause of the morphological changes in the macrophage cells, not the infection with *L. monocytogenes.* Compound SK-03-92 appears to be triggering some form of PCD in the macrophage cells. It is possible that this is occurring in the bacteria as well.

There are currently four commonly identified types of PCD: apoptosis, pyroptosis, autophagy/authophagic cell death, and necroptosis (82, 87). Apoptosis and pyroptosis are both pathways where the cell has directed itself to undergo death, albeit with different characteristics. Autophagy is typically considered a pro-survival pathway, but there is enough evidence that cell death can occur with autophagic markers that the term "autophagic cell death" has come to refer to the phenomenon whereby a cell is overwhelmed with autophagic vacuoles and ultimately the cell dies (82). Necroptosis is less cell-directed and more a result of environmental influences triggering a complex pathway that leads to cell death and release of intracellular content into the extracellular environment (88). The morphological hallmarks of these four types of PCD are described in Table 8.

The visible changes observed in this study in macrophage cells treated with 20 μg/ml of SK-03-92 for 5 h do not clearly point to any one type of PCD. There were morphological elements of multiple types of PCD: cellular enlargement, blebbing, vacuolization, lack of DNA fragmentation or chromatin condensation, intracellular content released to the extracellular medium, and cytoplasmic membrane permeabolization. It appears SK-03-92 is triggering some form or forms of PCD, but

TABLE 8 Types of programmed cell death and their morphological hallmarks

Type of programmed cell death	Morphological hallmarks (references)	Major effectors (references)
Apoptosis	DNA fragmentation, chromatin condensation, cell shrinkage, loss of membrane asymmetry, cytoplasmic blebs, apoptotic bodies, cells lose attachment, no release of internal contents to the extracellular environment (82, 89)	Caspases 2-3, 6-10 (82, 88)
Autophagic cell death	Massive vacuolization within the cell, degradation of cytoplasmic organelles, no chromatin condensation, no release of internal contents to the extracellular environment (82, 89)	LC3, ATG/Beclin proteins (82, 89)
Pyroptosis	Plasma membrane rupture, membrane vesicles, membrane re-sealing and swelling, nuclear condensation and rounding, balloon-shaped vesicle around the nucleus, loss of mitochondrial membrane potential, release of intracellular content to the extracellular environment (90, 91)	Caspase-1 (91, 91)
Necroptosis	Gain in cell volume, swelling organelles, plasma membrane rupture, release of intracellular content to the extracellular environment (88)	Kinases RIP1 and RIP3 (82, 88)

what these are remains to be determined. There are methods to test for the presence or activation of the major effectors of these major pathways. Further study could include investigation of which, if any, of the effectors are activated in treated mammalian cells.

Notably, resveratrol, the stilbene compound only two functional groups different from parent compound CL-3, induces autophagy through sirtuin-1 activation, which promotes cell survival (92, 93), not cell death as appears evident in the cells treated with SK-03-92. Other natural polyphenols such as pterostilbene and piceatannol have been shown to induce apoptosis in neutrophils (94), and, recently, another compound, stilbene 5c, was shown to arrest cell growth and cause cell death in tumor cell lines (95). Stilbenes and stilbenoid-like compounds have been shown to have an effect on PCD within human cells, adding credit to the hypothesis that SK-03-92 is behaving similarly.

There are systems in place for PCD in both prokaryotes and eukaryotes and it is possible that SK-03-92 may be binding to similar targets within both cell types. A similar phenomenon of increased PCD has been observed when treating bacteria (Rebecca Polanowski, personal communication) with SK-03-92. Microarray results (unpublished data, Rebecca Polanowski, personal communication) and other data suggest SK-03-92 may induce a stress response and activate PCD genes, factors known to impact autophagy. New research is revealing multiple PCD pathways in bacteria. Systems have been identified that have demonstrated cell death markers: *E. coli* toxin/antitoxin, prophage lysogeny, and the *S. aureus* Cid/Lrg system (83, 96). Biofilm formation in *B. subtilis* and *P. aeruginosa* are also under investigation regarding PCD and its impact on community survival (83). As well, many orthologs of the mammalian proteins involved in apoptosis and autophagy have been identified in yeast and studies are on-going to

clarify PCD pathways in these eukaryotic systems (78, 97). Further study may add to the hypothesis that SK-03-92 is impacting PCD in both prokaryotic and eukaryotic cells.

Exposure to SK-03-92 could possibly be activating the macrophages, which would also induce morphological changes such as enlargement and vacuole formation (98, 99). Typically, macrophage activation is due to triggering of pathogen-associated molecular patterns (PAMPs) binding to toll-like receptors (TLRs) on the macrophage cell surface. If SK-03-92 is binding TLRs and starting the activation signaling cascade, the macrophages would begin secreting cytokines to send for help. This is another avenue that could be explored in future work.

It was observed in this project that there is a cumulative effect of infection with WT *L. monocytogenes* in the cytosol of the macrophages and the presence of 20 μg/ml SK-03-92, leading to an increase in protein content at the 1 h time point. A logical supposition is that the increased protein content in the infected and treated cells was due to an increase in macrophage cell protein production. There are a number of possible reasons for increased protein production. When the endoplasmic reticulum (ER) undergoes stress, due to either pathogens or chemicals (100), the unfolded protein response (101) can lead to protein synthesis. The cell could also be increasing protein production in order to replace the membrane components used in making the phagosome that holds the foreign material, or the proteins may be the replacements for those in damaged organelles. There is evidence that SK-03-92 increases RNA synthesis in a dose-dependent manner (R. Ignasiak and W.R. Schwan, presented at the 70th Annual Meeting of the North Central Branch of the American Society for Microbiology, Mankato, MN, 1 to 2 October 2010), which could lead to increased protein content. Conversely, it is

possible that SK-03-92 is inhibiting proteasome activity, which could overwhelm the ER and the unfolded protein response with a buildup in misfolded proteins in the cell that then pushes the cell into ACD when trying to compensate, as was demonstrated recently with the bisbibenzyl agent Marchantin M (102). Whatever the target, SK-03-92 and WT *L. monocytogenes* together cause increased protein production at 1 h. This yields another piece of information helping to determine where and how SK-03-92 is binding within eukaryotic mammalian cells.

The bet-hedging phenomenon described above has been described in eukaryotic cells as well (103). When seeded at an initial concentration of approximately 5×10^5 cells/ml, about 45% of the macrophage cells remained attached to the well bottom and a third of these attached cells were still viable. Persisters are typically slowed metabolically and are in a dormant state. It is possible the macrophage cells were bet-hedging to protect themselves and that while the majority of the macrophage cells were affected by SK-03-92, populations persisted that promoted survival.

In conclusion, it was demonstrated that compounds SK-03-92 and VR-SK-03-92-2NITRO are able to penetrate human macrophage cells and inhibit growth of intracellular cytosolic *L. monocytogenes* within 5 h. The compounds do not kill bacteria within the phagosome during the 5 h window. These findings tell us the compounds are taken up by eukaryotic cells; however, the binding target is unknown. Also observed during this study were morphological aberrations caused by SK-03-92 in macrophage cells. The cells exhibited visible characteristics consistent with PCD, although the exact type of PCD and the signaling involved has yet to be determined. Overall, compound SK-03-92 is effective at killing cytosolic *L. monocytogenes*, but the effects on the macrophage cells need to be

further clarified in order to determine whether or not to pursue this antimicrobial for treatment of human disease.

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APPENDIX A

NIAID DATA

Appendix A. NIAID data

Data provided by NIAID following testing of compound SK-03-92 against *Mycobacterium tuberculosis* infected J774 murine macrophage cells

Compound	Log reduction[a] (0.5 µg/ml)	Log reduction (5 µg/ml)	Log reduction (50 µg/ml)
SK-03-92	2.26	2.23	4.37
Rifampin (positive control)	0.90	1.41	1.60

[a]Log reduction refers to the amount of bacteria present in SK-03-92-treated cells compared to untreated control cells 7 days post-infection.