Characterization of Novel Compounds that Inhibit Intracellular Salmonella Growth

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Abstract

Although instances of antibiotic resistance are steadily rising, traditional screening platforms have stopped producing novel antibiotics. The combination of these two events has led to fear that we may enter a post-antibiotic era, where common infections will no longer be treatable. In order to combat this issue, Abigail Reens and Amy Crooks in our lab carried out a high-throughput screen that identifies compounds that prevent bacterial growth in mammalian cells instead of in laboratory media. They screened a library of 14,400 drug-like compounds for activity against *Salmonella enterica* growing within macrophages. Of 286 chemicals with antibacterial activity, I found that only five have antimicrobial activity in standard laboratory media. Conditions inside the host cell are vastly different than those in broth. In cells, bacterial survival depends on virulence genes, which are often dispensable in standard media. Our screen therefore has the potential to identify compounds that target virulence genes or that synergize with conditions in the host to have antibacterial activity. We have begun characterizing the activity of our top 56 structurally unrelated hits and have found diverse and exciting results. Three hits appear to have previously undescribed activity against bacterial efflux-pumps, demonstrating the power of our approach to identify new chemical scaffolds with antibiotic potential. The hits I studied include one that is a member of a family of antidepressants with known intracellular antibacterial activity, five compounds that synergize with antimicrobial peptides, and one with antibacterial activity that is potentiated by incubation with macrophage supernatant.
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I. Introduction

a. Antibiotic Resistance is on the Rise

Antibiotic resistance is an urgent global health threat. The Centers for Disease Control and Prevention (CDC) estimates 23,000 people die annually in the United State of antibiotic resistant infections (Gross, 2013). Resistance among clinically relevant pathogens has been observed to almost every antibiotic available. *Clostridium difficile*, carbapenem-resistant *Enterobacteriaceae*, and MDR *Neisseria gonorrhoeae* have all been classified as urgent health threats by the CDC because they are often untreatable and have high mortality rates (Zilahi et al., 2016). Overuse and misuse of antibiotics has caused the incidence of antibiotic resistance to rise steadily, rapidly shrinking the window of time between antibiotic deployment and clinical resistance (Figure 1). In the past antibiotics could be used for years before there were cases of clinical resistance, but with today’s newest antibiotics that window has shrunk to mere months (Clatworthy et al., 2007).

**Figure 1**

Antibiotic deployment

![Antibiotic deployment chart](image)

*Antibiotic resistance observed*

*Figure 1: The time between antibiotic deployment and resistance is shrinking, while at the same time the discovery of new antibiotics is dwindling. These two factors have led to a large increase in multi-drug resistant (MDR) pathogens (Clatworthy et al., 2007).*
One factor that contributes to the rise of resistance is that all antibiotics prescribed today only target six essential bacterial processes (Payne et al., 2007). Furthermore, so-called new antibiotics are often structural analogs of these existing antibiotics. Therefore, bacteria often develop resistance to entire classes of antibiotics with the acquisition of a single mechanism. For example, β-lactams, such as penicillin and amoxicillin, inhibit cell wall synthesis. β-lactamase is a naturally occurring enzyme that confers resistance to β-lactams. Use of β-lactams by humans created a strong evolutionary force, selecting for pathogens that have acquired the β-lactamase gene and thereby evade antibiotic-mediated killing (Palumbi, 2001). There are in circulation some 20,000 resistance genes similar to β-lactamase, and excessive use of antibiotics has shaped bacterial populations so that only those containing resistance genes can survive (Liu and Pop, 2009). Despite the rising threat of antibiotic resistance, only two novel antibiotics have been discovered in the last 40 years (Clatworthy et al., 2007).

The decline in discovery of antibiotics is attributed to the exhaustion of traditional screening platforms and the unanticipated ineffectiveness of more modern approaches. Nearly all classes of antibiotics in use today were discovered between 1940 and 1960. During this “golden era of antibiotic discovery”, compounds were typically identified by screening soil-derived streptomycetes for antimicrobial activity (Lewis, 2013). This approach was so successful that by the mid-1960s it had been exploited to the point that discovery of novel antibiotics nearly ceased. In the early 2000s pharmaceutical companies attempted to revive antibiotic discovery by using target-based approaches (Payne et al., 2007). These took advantage of newly accessible genetic information. They began by identifying known, but underexploited bacterial targets, such as cell wall synthesis enzymes beyond the penicillin-binding proteins (PBPs), and designed compounds specific to that target. Genetic information was also used to identify highly conserved DNA sequences that could be used as potent broad-spectrum targets (Lewis, 2013). Despite the promise, target-based approaches failed to produce any antibiotics largely because they did not take into account the difficulty of getting a compound inside a bacterium.
and at high enough concentrations to bind its target effectively. Since the abandonment of target based approaches, antibiotic discovery has nearly come to a halt for lack of a solid platform.

In order to develop a new screening platform, we must think outside of the usual paradigm of antibiotic discovery. One thought that has driven antibiotic discovery since its dawn is that drugs must target ‘essential genes’ i.e. genes bacteria cannot live without. While this thought is still relevant, a close look at pathogenesis reveals what is unessential in nutrient rich broth, may become essential in the context of infection. All traditional screening methods took place in nutrient rich broth and therefore could not have identified genes that are only essential in the context of the host. This means ‘virulence genes’ or genes a pathogen depends on for survival in vivo are an entire class of unexploited, potential targets.

Considering virulence genes targets also reflects a paradigm shift in how the medical community sees bacteria. When the first antibiotics were designed their goal was to be broad spectrum. Since then, there has been a lot of evidence that the health of the human microbiome is a huge contributing factor to health overall (Huttenhower et al., 2012). This has changed the way antibiotics are seen. It is no longer thought that broad spectrum antibiotics are the most rational design, because preserving the microbiome is one of the most efficient ways to prevent opportunistic infection (Tosh and McDonald, 2012). Changing the way antibiotics are designed to reflect this shift in attitude would modernize a technology that has not been changed since its discovery.

a. *S. enterica* Pathogenesis and Virulence Genes as Potential Targets

The complexity of *S. enterica* pathogenesis reveals the multitude of potential targets for novel antibiotics, many of which are already proven to be lethal in knock-out mutants (Fields et al., 1986).

*S. enterica* enters the gut through or ingestion then traverses the highly acidic environment of the stomach to reach the small intestine where it can cross the endothelium and gain entrance to the underlying tissue. Survival in the stomach and later the Salmonella Containing Vacuole (SCV) depends on
the expression of two distinct sets of Acid Tolerance Response (ATR) genes. Mild acidic conditions (pH = 5.8) induce the expression of the log-phase ATR genes. Induction maintains homeostasis when constitutively expressed homeostasis genes are no longer sufficient. While this set of ATR genes is switched on, *S. enterica* continues to replicate normally. When the environmental pH reaches below 4.5, stationary phase ATR genes are activated. These genes code for 43 proteins that prevent and repair damage from exposure to extremely low pH. During acid shock, *S. enterica* enters stationary phase and does not replicate. Both sets of these genes are necessary for survival inside the stomach (pH 1.5 to 3.5) and inside the phagolysosome (pH 4.5-5) (Geisow, 1981; Wilmes-Riesenberg et al., 1996). This means a compound that targets any of the major genes in the two ATR sets could be lethal *in vivo* and have no phenotype in standard, nutrient-rich broth.

Next *S. enterica* arrives at the small intestine where it will cross the microvilli to enter the rest of the body. Although the pH of the small intestine is more hospitable, *S. enterica* must evade digestive enzymes, IgA, bile salts, and antimicrobial peptides (AMPs) by expressing more virulence genes (Haraga et al., 2008). Many of these virulence genes are controlled by the PhoP-PhoQ two component regulatory system. PhoQ is an inner membrane sensor that senses low magnesium, a common feature of stressful environments. It phosphorylates PhoP when it senses low magnesium, activating it to act as a transcription factor for virulence genes (Groisman, 2001). The resistance of *S. enterica* to bile salts and AMPs are dependent on the PhoP-PhoQ system (Prouty et al., 2004). Mutations in 83 virulence genes, many of them controlled by the PhoP-PhoQ, were shown to be lethal to *S. enterica* in macrophages and *in vivo* in mice, despite the fact all mutants could grow in LB (Fields et al., 1986). This work supports the theory that targeting virulence genes is a viable strategy for designing novel antibiotics.

Finally, *S. enterica* must exit the small intestine to enter the periphery and eventually its niche in the SCV. There are four known mechanisms by which *S. enterica* can gain access to the SCV. The first mechanism is via bacterial mediated endocytosis. Here *S. enterica* adheres to the apical surface of
epithelial cells using fimbrial adhesions and induces membrane ruffles. The membrane ruffles engulf \textit{S. enterica}, allowing it to pass through. Another method of traversing the epithelium is \textit{S. enterica} can disrupt tight junctions between endothelial cells allowing for the flow of excess fluid into the lumen. \textit{S. enterica} also crosses the epithelium via M cells, which are specialized cells that take samples from the lumen to present to Peyer’s patches. It has been shown \textit{S. enterica} preferentially enters via M cells because that brings it directly to lymphoid tissue, where it can access its niche quickly (Jones, 1994).

Finally, \textit{S. enterica} can cross the epithelium if it is phagocytosed and transmigrates with the professional phagocyte (Vazquez-Torres et al., 1999). \textit{S. enterica} cannot induce morphological changes in the host cell cytoskeleton without the expression of virulence genes induced by stress conditions like low O$_2$ (Francis et al., 1992). So all of these mechanisms of traversing the epithelium are dependent on the ability of \textit{S. enterica} to sense its environment and induce physiological changes at the right moment. If any of the genes required to do so could be inhibited by a drug it is highly possible \textit{S. enterica} would be rendered avirulent.

Once across the epithelium \textit{S. enterica} is phagocytosed by resident macrophages it lives and replicates in the SCV. Although this is its niche, it is still a harsh environment where virulence genes are crucial for survival.

\textbf{b. Novel Screen for Antimicrobials Exploits the Unique Environment of the SCV}

Inside the macrophage \textit{S. enterica} faces many of the same challenges it faced throughout the GI tract- harsh acid, AMPs, low magnesium, low iron, and lack of nutrients. In order to thrive in this harsh environment \textit{S. enterica} must express more virulence genes.

Taking advantage of the fact \textit{S. enterica} depends on virulence genes for survival Abigail Reens, a Ph.D. candidate in the Detweiler Lab, with help from Amy Crooks, a research technician, developed a high-throughput, fluorescence based platform to identify small molecules that have anti-microbial activity against intracellular bacteria. This approach has the potential to identify compounds that target
virulence genes because they are essential in the context of the SCV. The platform may therefore identify potential antibiotics that were missed by screening in nutrient rich laboratory media. The screen also avoids compounds that cannot reach the bacteria in an active form or that are toxic to mammalian cells, two major advantages over target-based approaches. The identification of potential antibiotics that access Gram-negative pathogens is especially important because the outer membrane characteristic of Gram-negatives poses an extra challenge for antibiotics (Silver, 2011).

The screening platform uses microscopy images of the host-pathogen model system to quantitatively identify compounds that decrease GFP-expressing *S. enterica* load without reducing macrophage vitality. Vitality is quantified using MitoTracker Red, which localizes to active mitochondria based on membrane potential (Kholmukhamedov et al., 2013). Overall the screen has the potential to find novel antibiotics that are relevant the niche of *S. enterica* during infection.
II. Results

a. Hits With Antibacterial in Cells But Not in Broth

Amy Crooks and Abigail Reens screened the 14,400-compound Maybridge Hitfinder™ Collection v11 and found 309 hits that reduced *S. enterica* load but did not impact macrophage vitality. These hits were confirmed by colony forming unit (CFU) assay. The activity of the compounds was then ranked based on percent inhibition in the GFP-MT assay, percent inhibition in the CFU, and consistency. If a compound did not validate in the CFU it was dropped from this study. CFU assays are the field standard to quantify bacterial load of an infected macrophage. Because our screen is novel it was important its results could be corroborated by established methods. We identified 286 compounds (hits) that decreased bacterial load consistently in both GFP-MT and CFU (data not shown) and gave them ranked identifiers DET1-286.

Once hit compounds had been identified and cherry-picked, I tested the top 60 hits in rich broth at the screening concentration of 25 μM. At this concentration, only DET5 inhibited *Salmonella* growth in the rich broth condition (Figure 1A). DET5 is the known, broad-spectrum antibiotic, chloramphenicol which is used clinically to treat *S. enterica* in some sensitive and MDR cases (Asperilla et al., 1990). Since its *in vitro* and *in vivo* activities are both well classified, chloramphenicol served as an internal positive control.

After I found none of our top 60 hits had activity at the screen concentration in rich broth, I decided to expand our broth analysis to test all 286 hits against *S. enterica* and raise the concentration to 100 μM. I still only found 3 compounds that inhibited growth: DET5, DET58, and DET78. DET58 and DET78 both reduced growth by about 30% (Figure 2B). Figure 2C shows exemplary growth curves for *S. enterica* in LB in the presence of various 100 μM compounds. DET186 is a compound that clearly had no impact on *S. enterica*, and is representative of what *S. enterica* growth looked like in the presence of the vast majority of our hits. This was an incredible result that demonstrated the stark differences between
intracellular and rich broth conditions. Genes that are completely required for life in the SCV, may serve little or no importance in rich broth where there is no stress. This result also emphasized the powerful potential of our screen to identify targets all previous broth-based screening methods would have missed.

Figure 2

Figure 2: Before repurchase 3/286 hits from the screen had activity in broth LB. *S. enterica* was grown in LB in the presence of a hit at either 25 or 100 μM. A and B are snapshots of the optical density at the 16 hour time point. DMSO, the vehicle control, is the first point on each graph. A shows the top 60 hits, tested at the screening concentration, 25 μM n=1. B shows all 286 hits from the screen tested at 100 μM n=1. C is exemplary growth curves of *S. enterica* grown for 16 hours in the presence of compounds of interest. DET186 is a representative compound with no activity n=1.

**DET58** displayed moderate antimicrobial activity and is highly similar to known antibiotics nitrofurantoin and furazolidone (Figure 3). The mechanism of these antibiotics is poorly understood, but it is commonly believed these compounds are active because of their break down product: nitrenes (M. Barbachyn). Susceptibility to these drugs correlates with the presence of nitroreductases, which are present in *S. enterica*, but are primarily induced under oxidative stress (McOsker and Fitzpatrick, 1994; Nokhbeh et al., 2002). While DET58 only inhibited growth by 30% in broth, it inhibited growth by 88% in
the GFP-MT assay (data not shown). This supports DET58 may depend on the presence of nitroreductases because there is oxidative stress in the SCV but not in LB broth. These compounds are also known to be mutagens, so we did not continue study of DET58.

**Figure 3**

![DET58 molecule](image)

**Figure 3**: DET58 is structurally similar to nitrofurans, an established family of antibiotics. Image and insight courtesy of Mike Barbachyn.

DET78 is a known topical antiseptic, 9-aminoacridine (2016a). It is a mutagen and has been shown to produce frame shift mutations in E. coli (Acharya et al., 2007). Due to its mutagenic activity it has come up in many common yeast screens for anticancer drugs (2016a). The ability to mutagenize both bacterial and eukaryotic cells makes it a very unattractive antiseptic and it is not currently in clinical use. Like DET58, after this broth assay we chose to drop DET78 from further studies.

The initial broth assays confirmed our screen is a valid way to identify potential antibiotics. There were three compounds that showed activity in rich broth. One was a known antibiotic, one was structurally similar to known antibiotics, and the third was a known antiseptic. All were positive indicators of both assays’ function. The identification of no other compounds with broth activity demonstrated our screen was highly specific for finding compounds that kill *S. enterica* in the SCV.
Following the initial broth assay Jessy Podoll, a post-doc in our lab, examined the structures of our hits to see if there were any patterns or motifs. She found the majority of the hits clustered into 13 categories or families. We repurchased the best one or two compounds from each cluster to maximize the diversity of the 60 compounds we would continue to study. All of the compounds we repurchased had a minimum of 70% inhibition intracellularly (data not shown). After reordering, we confirmed the compounds were all still active in the GFP-MT assay, and then I tested them in broth. Corroborating our original data, the majority were still inactive in broth (Figure 4).

Figure 4

Figure 4: After repurchase of the top 56 structurally diverse hits 3/56 had activity in MHB. S. enterica was grown in the presence of the top 56 structurally diverse hits after repurchase at a concentration of either 25 or 100 μM. Error bars represent SEM. If they are too small, they are hidden by the dot and not visible. A and B are snapshots of the optical density at the 18 hour time point. DMSO, the vehicle control, is the first point on each graph. Each other dot represents a compound tested at 25 or 100 μM respectively n=2. C and D represent 18 hour growth curves of S. enterica in the presence of hits of interest. DET2 and 3 represent compounds with no broth phenotype like 53/56 compounds n=2.
At 25 μM there were still no active compounds, except chloramphenicol (Figure 4A). At 100 μM two compounds DET30 and 35 were active (Figure 4B). These two did not show up in the original broth assays, so that may be an indication they experience chemical breakdown. The plates used in the original assays went through 10+ freeze thaw cycles- which could cause unstable compounds to break down, explaining why the repurchased compound inhibited bacterial growth while old freeze-thawed compound did not. DET30 and 35, later came up in the Hoechst assay which identifies efflux pump modulators (EPMs) and their mechanisms are still being investigated (A. Crooks and A. Reens). One way *S. enterica* protects itself from many insults in the SCV is efflux. For example, the AcrAB–TolC system which forms an efflux pump that is required for pathogenesis. *AcrAB-TolC* mutants are more sensitive to low pH, bile and many antibiotics. They also are unable to infect macrophages and do not persist in the gut *in vivo* (Buckley et al., 2006). While efflux pumps serve a small purpose in broth, they are essential for life *in vivo* where there are more threats to pump out. This may explain why we did not see any effect of the efflux pump drugs at 25 μM in broth, but we did in macrophage. The fact that these compounds were active at 100 μM, contributed to our hypothesis that they were acting on *S. enterica* directly.

Even if efflux pump modulating drugs are not sufficient to completely kill bacteria on their own, they can be used to resensitize resistant bacteria to commonly used antibiotics. One of the main resistance mechanisms *S. enterica* uses to antibiotics is it upregulates its efflux pumps and pumps the drug out. It has been shown, fluoroquinolone- resistant *S. enterica* can be resensitized to ciprofloxacin and other fluoroquinolones if their efflux pumps are mutated (Soto, 2003). This means any new EPM drugs could be used in concert with currently prescribed antibiotics in clinical cases where resistance is a problem.

After repurchase of the top 60 structurally unrelated compounds, our original conclusions were confirmed. The vast majority of the compounds were still highly active in the GFP-MT assay and not
active in broth. The secondary broth assays also exposed some compounds that may be likely to breakdown and lose activity.

b. Top Hit and Structurally Related Compounds Inactive in Broth

Interestingly, the top compound that came out of our screen was a FDA-approved tricyclic antidepressant (TCA), clomipromine. Clomipramine inhibited 100% of *S. enterica* replication in the GFP-MT assay, but like many compounds had no activity in broth at 25 or 100 μM. In order to determine structure-activity relationships (SAR), we purchased eight TCAs that were structurally related to clomipramine (A. Reens). All the TCAs had similar IC50s to clomipramine in macrophages and in broth (A. Reens and M. Edwards) (Figure 5). I tested their broth activity according to the *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition*. In tandem with all of the TCAs I tested the MIC of ampicillin as a control. Ampicillin has a reported MIC of <14 μM in SL1344 (Wikler et al.). I determined the MIC to be between 8 and 16 μM, establishing our protocol as effective. None of the TCAs had an MIC under 300 μM. Clomipramine and structurally related drugs clearly have no activity in broth despite having activity at antibiotic-comparable IC50s in macrophages. Abigail Reens is still investigating how these TCAs are such potent antimicrobials intracellularly.
Drugs that synergize with antimicrobial peptides (AMPs) are of particular interest because studies have shown these drugs have no efficacy in broth, but are excellent antibiotics in vivo even against MDR pathogens (Lin et al., 2015; Sakoulas et al., 2014). It has been shown AMPs, like LL-37 can permeabilize the membranes of MDR pathogens, resensitizing them to common antibiotics (Lin et al., 2015). LL-37 competitively displaces Ca+ and Mg+ in LPS, then forms a toroidal pore in the phospholipid bilayer (Henzler Wildman et al., 2003; Turner et al., 1998). This pore has been shown to allow drugs like azithromycin to accumulate inside MDR pathogens lethal levels (Lin et al., 2015).

AMPs are a class of proteins that have broad-spectrum effects directed at aiding a host in eliminating a pathogen. They are short peptides, highly conserved across all species that have activity against gram(+) and (-) bacteria, fungi, parasites and viruses (Thomas et al., 2010; Zasloff, 2002). They
can be broken down into structural families though most are cationic and amphipathic (Khamis et al., 2015). These properties allow AMPs to interact with the membranes of microbes and permeablize them. They often depend on the zwitterionic characteristics and low cholesterol of pathogen’s membranes to differentiate them from self (Henzler Wildman et al., 2003). Membrane disruption is best classified AMP activity, but it has also been shown they disrupt many other biological processes and are pleiotrophic (Chen et al., 2012).

I tested synergy of the hits from the screen with LL-37. Synergy was tested in the presence of 5 μg/mL LL-37 because this is 50% of its MIC (Figure 6A). Drugs were tested at a single concentration, 50 μM. Of the repurchased compounds, five synergized with LL-37 and fully inhibited growth, including the two EPMs (Figure 6B). Two of the other compounds that inhibited growth in the presence of LL-37, DET22 and DET71 bother appeared to be bactericidal. Growth in the presence of either of these compounds and LL-37 was normal at first, and then completely dropped off around the 7 hour mark. Many more partially inhibited growth or caused delayed lag phases (data not shown).

**Figure 6**

**Figure 6**: Five compounds synergized with LL-37 and completed inhibited bacterial growth in its presence. *S. enterica* was grown in minimal media supplemented with LL-37. A shows the MIC of LL-37 for *S. enterica* in minimal media was 10 μg/mL. Based on this number, minimal media was supplemented with 5 μg/mL LL-37, 50% of the MIC to test all repurchased hits n=3. B shows representative curves of the five hits that synergized with LL-37. Of those 5 hits, DET21 and 71 look bactericidal. In the presence of DET27 and 71 *S. enterica* grows normally for 7 hours then rapidly drops off. Two of the other compounds that synergized with LL-37 were the EPMs n=2.
d. Macrophage Supernatant Potentiates the Activity of DET88

In parallel with the LL-37 experiments I also tried to elucidate if anything else the macrophage produced potentiated these drugs into having antibiotic activity. My idea was to grow bacteria in a condition that was more similar to the screen, if I could find a condition that a compound inhibited bacterial growth in I could use that condition to determine a mechanism. In order to do this, I either collected the supernatant from macrophages or used LPS to stimulate macrophages then after 20 hours collected the supernatant. This gave me two types of supernatant: unstimulated and stimulated. I used the supernatant supplemented with LB as a media to test the efficacy of the compounds. LPS is a major component of the outermembrane of gram-negative bacteria. LPS is recognized by macrophages via toll-like receptor 4 (TLR4) (Parham, 2015). This initiates a kinase cascade that eventually leads to the release of the transcription factor NFκB, which is stored in the cytosol in an inactive form. NFκB then initiates the transcription of genes that aid in the fight against infection. These genes include, but are not limited to cytokines, immunoreceptors, acute phase proteins, and stress response genes (Gilmore, 2016).

All compounds were tested at 50 μM. Only one compound appeared to have activity in the macrophage supernatant, DET88, but the results are still being actively investigated. It had a clear phenotype in cell culture, but lost it after a few experiments (Figure 7 and 8A). Interestingly, Toni Nagy, a post-doc in our lab also showed it was as effective as chloramphenicol in mice in one experiment, and had no activity in another (data not shown). We are still determining what conditions influence the activity of this compound and why its phenotype was lost in two different types of experiments after being so strong.

In the experiments where DET88 was active, it inhibited S. enterica growth by about 50% at 50 μM. This was striking considering it had no activity at 100 μM in rich broth (Figure 2B and 4B). DET88 was slightly more active in when tested in supernatant that came from LPS stimulated macrophages (50% inhibition). In the supernatant from macrophages that had not been exposed to LPS, it inhibited growth
The difference in percent inhibition was only about 10% at the 18 hour time point (Figure 7C). However, the log phases of the two graphs look completely different. When the macrophages were not LPS stimulated, the log phase looks very similar to the positive control. In the supernatant that came from LPS stimulated macrophages, the slope of the log phase is greatly reduced (Figure 7A and 7B). This led us to hypothesize that DET88 synergizes with something that is constitutively expressed, but upregulated via LPS dependent signaling.

**Figure 7**

Prior to losing DET88 activity we had been using a plate that had been freeze-thawed many times for other assays. We stopped keeping compounds in the freezer and started just storing them at room temperature in the dark when we realized some were more stable in conditions where the temperature wasn’t changed frequently. When we refilled that plate from our stock that had not been
freeze-thawed, we lost activity. This made us wonder if the part of the molecule responsible for the activity actually needed to be released as a breakdown product or as a result of an enzyme. To test this we took small aliquots of DET88 and freeze thawed them various amounts of time between 0 and 20.

Even after 20 freeze thaws we did not regain activity, so this hypothesis was disproven (Figure 8b).

**Figure 8**

![Figure 8](image)

Figure 8: DET88 lost its phenotype in macrophage supernatant and no phenotype was regained during a decomposition assay. A and B are both growth curves of *S. enterica* in LPS stimulated supernatant with 50 μM compound. Error bars represent standard deviation and technical replicates are shown. A shows DET88 lost its phenotype and looked the exact same as a compound with no activity n=2. B shows DET88 after varying number of freeze-thaw (FT) cycles. Activity was never regained, so a chemical break down product of DET88 is not responsible for its activity n=1.
III. Conclusions and Future Directions

Overall, our intracellular screen emphasized the value of a host-pathogen model for antibiotic discovery. Results from our efforts proved that screening in traditional nutrient rich broth is not always indicative of which compounds will be efficacious in the host. All of our hits had excellent antimicrobial activity intracellularly, but very few had any activity in broth. The compounds that did have activity in rich broth were excellent indicators that our screen identified relevant compounds. DET5 is a known antibiotic, DET58 is structurally similar to known antibiotics, DET78 is a known antiseptic, and DET35 and DET43 are efflux pump modulators (EPMs). This is also evidence that our screen could identify compounds that target virulence genes. Virulence genes are not essential to life in nutrient rich media, but are essential in the stressful conditions of the host. One example we observed were the EPMs. Bacteria including *Salmonella* increase expression of efflux pumps when faced with stressful conditions. These enable the bacterium to protect themselves from insults by exporting threats. Therefore inhibiting efflux pumps can be deadly to *S. enterica* when it is in the SCV, despite only having a small effect in broth. The ability to identify virulence genes as targets sets this method apart from traditional screening methods where compounds that target virulence genes would be missed. This means there are a large number of unexploited targets, theoretically different than their predecessors, that could usher in a new era of antibiotics. Although many scientists were equally as hopeful about target-based approaches, those methods proved ineffective. This is due to the fact target-based approaches did not take into account the complexity of getting a compound into the bacterium and localizing to the right area at a high enough concentration to actually bind a target. Our screen inherently overcomes the issues that plagued target-based approaches. Compounds that cannot accumulate at high enough levels in the bacterium are automatically eliminated. Our screen also takes trafficking and accumulation issues a step further because not only does it ensure the compound can
accumulate inside the bacterium, but it also requires that compounds traffic to the appropriate vesicle inside the macrophage without obviously impacting vitality.

The top hit from our screen is another example of a compound that would go unnoticed in traditional antibiotic assays. Clomipramine is an FDA-approved tricyclic antidepressant (TCA) used clinically to treat depression and obsessive compulsive disorder. Because clomipramine is already FDA-approved there is a lot of information available about its toxicity and off target effects that could be used in developing it as an antibiotic. However, even if it could not be used as an antibiotic it could lead to novel insight on the interconnection between neurotransmitters and the immune system. This is the second intracellular screen in macrophages to identify a psychoactive drug out as a top hit, so there is clearly an underlying relationship that has not been defined. A similar result was found in 2009 against *Listeria* but the mechanism was never fully elucidated (Lieberman and Higgins, 2009). It has long been known that neurological disorders like depression and anxiety can lead to chronic pain and higher likelihood for infection (Katon and Ciechanowski, 2002). Clomipramine and other psychoactive drugs with antibacterial activity could be used as tools to examine the molecular mechanisms that connect mental health to immunity and inflammation.

Another interesting group of hits from our screen are those that synergize with the human antimicrobial peptide LL-37. DET27, 35, 43, 71, and 83 had very clear phenotypes and fully inhibited *S. enterica* growth at a concentration less than half of their MIC in rich broth. Based on the significant difference in these five compounds’ MICs in the presence or absence of LL-37, I hypothesize that they interact with the antimicrobial peptide in a synergistic manner and should be further characterized. Compounds that synergize with host antimicrobial peptides would be advantageous because other studies have shown compounds that synergize with LL-37 are effective against MDR pathogens *in vivo* even if they have no standard broth phenotype (Lin et al., 2015; Sakoulas et al., 2014). In these papers it was shown LL-37 permeabilizes the membrane allowing a drug to enter and accumulate. It would be
interesting to test and see whether this is the method of synergy is being used with our hits. It could be easily done with florescence microscopy and would an interesting project for someone in our lab in the future to pursue. Testing would involve staining the bacterial membrane with a fluorescent dye for example FM4-64 (red) and treating the bacteria with a florescent analog of the drug of interest in the presence or absence of the antimicrobial peptide. Increased accumulation of the florescent drug only in the presence of LL-37 would indicate that LL-37 permits the drug to enter the bacterium where the drug likely acts as an antibiotic. If the drug accumulates inside the bacterium in the presence of a non-ionic detergent (eg Triton X-100), but does not maintain a low MIC, that would indicate that the antimicrobial activity depends on different or multiple functions of LL-37 (Lin et al., 2015).

Another way to gather evidence about this issue would be to test compounds that require LL-37 against a gram-positive pathogen in broth. Gram-positive pathogens have less of a barrier that drugs need to cross to enter the cell, so some drugs that depend on permeablization in gram-negatives may work independently in gram-positives (Silver, 2011). This wouldn’t be a definitive test because there could be another array of reasons the drug doesn’t work in gram-positives, but it could help corroborate other evidence. Finding antimicrobials that work in the presence of AMPs could expand the arsenal of antibiotics available, and thus help fight antibiotic resistance. Drugs that only work in the presence of antimicrobials may also be of interest because they would not continue to influence bacterial genetics once deposited into the environment in the absence of AMPs. This could help slow resistance while simultaneously creating novel drugs.

One compound I identified, DET 88, possessed some antibacterial activity in supernatant from LPS-stimulated macrophages. Although this compound showed promise initially, the phenotype originally obtained was lost. In order to understand the root cause of DET 88 activity loss, we will need to take steps to understand whether there are structural changes to the compound that occur during storage or freeze-thaw cycles. Such measures will include running Mass-Spectrometry (MS) on different
samples of the compound we have been using and then determining storage conditions in which little breakdown occurs.

If we regain the DET88 phenotype there are also some follow up experiments we could do to study its mechanism of action. Because it appeared DET88 synergized with some product of macrophage LPS stimulation, the downstream products of this signaling pathway could be examined for their synergistic effect on DET 88 activity. This would give insight into potential products of this pathway that could be used to potentiate antibiotics.

Interestingly, DET 88 has 81% structural similarity to a compound that has a known target in *E. coli* (Figure 9) (2016). This compound binds the enzyme 2-C-methyl-D-erythritol (CDP-ME) kinase, which is necessary for the synthesis of isoprenoids. Isoprenoids are a class of molecule that are essential for all forms of life (Heuston et al., 2012). An example of a widely known isoprenoid is ubiquinone, an electron carrier in the electron transport chain. There are two pathways that synthesize isoprenoids. Eukaryotes, archaea, gram-positive bacteria and use the mevalonate pathway, while gram-negative bacteria use the methylerythritol phosphate (MEP) pathway (Lange et al., 2000). CDP-ME is an enzyme in the MEP pathway, making it a highly attractive novel antibiotic target because currently no antibiotics that target any enzyme in this pathway and it is unique to gram-negatives (Tang et al., 2011). It has also already been shown that genetic knock outs to any enzyme in the MEP pathway are lethal to *S. enterica* (Cornish et al., 2006).
It would be interesting to see whether DET88 has a similar mechanism of action to this related compound and there are many ways to test if this is the case. If DET 88 is effective against *E. coli*, its efficacy could be compared to the similar compound. In addition the compound similar to DET 88 could be run through our GFP-MT screen and against *S. enterica* in broth and in the supplemented broth from the macrophage experiments. This would provide initial structure-activity relationship data that could aid in the determination of the molecular target of DET 88. If the counterpart of DET 88 is not active in macrophages, it is possible that the part of the molecule they do not share helps DET 88 enter the macrophage and traffic to the SCV. If DET 88 and the compound similar to it both have no activity in gram-positive bacteria that would indicate they target a pathway gram-positives do not have, like the MEP pathway.

If the previous assays were to produce results that made it appear DET88 does target CDP-ME, CDP-ME could be purified from *E. coli* that over express it and a binding assay could be done. An *E. coli*
already exists that over expresses CDP-ME with a his-tag so it could be purified with an affinity chromatography column (Boucher and Doolittle, 2000).

The Detweiler lab continues to make progress toward identifying the pathways targeted by compounds identified in our screening effort. It has been my goal to aid in the initial characterization of these compounds and to set the stage for continued research efforts. These data make it very clear that intracellular screens could be a highly effective platform for discovery of novel antibiotics.
**IV. Materials and Methods**

**Bacterial Strains and Growth.** *Salmonella enterica* (*S. enterica*) serotype typhi strain SL1344 was used for all experiments. It induces typhoid fever symptoms in mice and is resistant to streptomycin (Hoiseth and Stocker, 1981). Overnight cultures were grown in LB and streptomycin. Compounds were ordered from the Maybridge Hitfinder™ Collection v11 and resuspended in DMSO.

**Single Concentration Broth Activity.** Overnight *S. enterica* culture was washed three times in PBS then resuspended in either Luria-Bertani (LB) broth or Mueller-Hinton broth (MHB) at a final concentration of 0.01 OD600. Compounds were added at either 25 μM or 100 μM. The concentration of DMSO per well was never greater than 1%. The plates were then grown at 37°C for 18 hours and growth was monitored with the BioTek Eon incubator shaker microplate absorbance reader.

**MIC.** The minimum inhibitory concentration (MIC) of the ampicillin, the TCAs, and LL-37 against *S. enterica* were found using methods adapted from *Performance standards for antimicrobial susceptibility testing*. For the MIC of ampicillin and the TCAs, *S. enterica* was grown 18hrs in LB, washed 3x in PBS and resuspended in MHB at an OD600 of 0.01. For the ampicillin MIC, ampicillin was diluted from a stock to the highest concentration (32 μM), then diluted in MHB two fold down to 4 μM. For the TCAs, all were diluted from the stock to the highest concentration tested (1000 μM), then diluted in MHB 1.5 fold down to the lowest concentration tested (222 μM). Not all of the TCAs were soluble in MHB at 1000 μM, so for some the highest concentration was 750 μM. Once the plates were loaded with media, drugs, and *S. enterica* they were grown overnight at 37°C in the BioTek Eon incubator shaker microplate absorbance reader and growth was monitored.

**Synergy with LL-37.** For experiments testing drug synergy with LL-37, *S. enterica* was washed three times in PBS then resuspended in minimal media. The MIC of LL-37 (10 μg/mL), used to decide a testing concentration for synergy, using method described above. Minimal media consists of 20% M9 salts (211.3 mM Na$_2$HPO$_4$, 110 mM KH$_2$PO$_4$, 93.4 mM NH$_4$Cl, 42.77 mM NaCl), 0.004% histidine, 1 mM
MgSO₄, 20% dextrose and water. LL-37 was used at a final concentration of 5 μg/mL, 50% of the MIC90. Hits from the screen were tested in the minimal supplemented LL-37 at 50 μM. The growth was monitored overnight at 37°C in the BioTek Eon incubator shaker microplate.

**Cell Culture.** An immortalized murine macrophage cell line264.7 (RAWs) was used for all cell culture experiments. This cell line lacks functional NRAMP-1, so *S. enterica* has access to iron and replicates well. This is a common model for *S. enterica* infection (Raschke et al., 1978). RAWs were cultured in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol. They were grown at 37°C with 5% CO₂.

**Synergy with Supernatant.** For experiments testing drug synergy with peptides made by RAWs, they were grown to confluence (3-5 days) then reseeded at a concentration of 4E5 to 5E5 macrophages per mL. After reseeding they were allowed to rest for 2 hours then stimulated with 20 ng/mL lipopolysaccharide (LPS) or vehicle control and left in the incubator. After 20 hours supernatant was collected and used immediately, supplemented with 5% LB to improve bacterial growth. *S. enterica* was grown overnight for 18 hours, washed and resuspended at 0.01 OD600 in LB. All drugs were tested at 50 μM, making the final concentration of DMSO 0.5% of the well. Growth in this media was monitored by the BioTek Eon incubator shaker microplate absorbance reader for 18 hours.
V. References


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