ABSTRACT

Title of Document: PROPERTIES OF A GALLIUM-CONJUGATED SIDEROPHORE COMPLEX AS AN ANTIBACTERIAL TREATMENT

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In 2013, the Centers for Disease Control and Prevention estimated that approximately two million individuals in the United States developed antibiotic-resistant infections. This increasing rate of bacterial antibiotic resistance necessitates the development of alternative treatments. Gallium-desferrioxamine (Ga-DFO), a gallium-chelated bacterial siderophore, offers a promising alternative treatment by exploiting the natural iron-uptake pathway of bacteria to introduce toxic gallium ions into the cytoplasm. Previous research demonstrates that the Ga-DFO complex is effective against a number of bacterial strains in ideal treatment conditions. The present study examines the properties of Ga-DFO and its effectiveness against additional strains in a non-iron-depleted environment and in mammalian cell culture. Ga-DFO was found to successfully inhibit the growth of each bacterial strain tested. It was also effective when added at any time point during bacterial growth.

PROPERTIES OF A GALLIUM-CONJUGATED SIDEROPHORE COMPLEX AS AN ANTIBACTERIAL TREATMENT

By

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Properties of a Gallium-Conjugated Siderophore Complex as an Antibacterial Treatment

The introduction of antimicrobials transformed the world of medicine. Initially, this new class of drugs treated formerly lethal infections with ease. Unfortunately, widespread use of antibiotics has created a global selection pressure for antimicrobial-resistant phenotypes that threaten our current paradigm of disease treatment.

A 2014 global analysis of antibiotic usage estimated a 36% increase in antibiotic consumption from 2000 to 2010. Anti-*Escherichia coli* antibiotics (fluoroquinolones and cephalosporins) were among the largest absolute increases, of 8.1 billion and 3 billion standard units respectively (Van Boeckel, et al., 2014). Unsurprisingly, there has also been an increase in the rate of drug resistant *E. coli* infection in humans (Pitout & Laupland, 2008). There is strong evidence the use of antibiotics to supplement livestock has contributed to human acquisition of these drug-resistant strains. They do so by encouraging the development of resistant strains in livestock which are subsequently ingested by humans via diet (Corpet, 1988). There is consensus amongst researchers that antibiotic overuse should be curbed to prevent long term spread of antibiotic resistance (Collignon, 2009; Wintersdorff et al., 2016), while more immediate solutions include developing alternative antibacterial treatments.

E. coli has a well studied mechanisms of resistance to traditional antibiotics such as cephalosporins and fluoroquinolones. These include the alteration of drug targets, mutations to affect drug accumulation, and expression of drug-targeting plasmids (Jacoby, 2005). These mechanisms work synergistically to contribute to antibiotic resistance, and the inhibition of one mechanism can lead to recovery of antibiotic susceptibility (Jacoby, 2005). In this study, we investigate a novel antibiotic treatment that specifically bypasses *E. coli* strategies to inactivate

drugs and prevent drug accumulation, with broader possible applications against other Gram-negative and Gram-positive bacteria.

One strategy of drug resistance used by *E. coli* is prevention of drug accumulation. *E. coli* and other Gram-negative bacteria are able to regulate influx and efflux of extracellular materials via expression of outer-membrane porins and efflux pumps, respectively (Nikaido, Rosenburg, Foulds, 1983). Outer-membrane porins selectively allow extracellular materials to enter the cell, while efflux pumps actively pump out undesirable materials from the cytoplasm. Low outer membrane permeability, coupled synergistically with an efflux system, prevent the intracellular accumulation of antibiotics (Fernandez & Hancock, 2012). This defense may be bypassed by hijacking an essential uptake pathway.

The siderophore iron-uptake pathway is a prime candidate for manipulation. Siderophores are small iron-chelating molecules that bacteria release into their environment to scavenge iron and return to the bacterial cell (Neilands, 1995). This creates an ideal situation for input of an antibacterial substance that uses a "Trojan Horse" approach to infiltrate the cell. Since iron is essential to many metabolic processes, this iron-scavenging pathway is crucial for bacterial survival (Pierre, Fontecave, & Chrichton, 2002). Therefore, any adaptations that would decrease the activity of the pathway would also decrease the amount of iron uptake, potentially leading to cell death.

In addition, many bacteria possess receptors for siderophores produced by other organisms, and are able to uptake foreign siderophores in order to maximize their iron uptake in a phenomenon called siderophore piracy (Luckey et al., 1972). Studies suggest that bacteria may need to use siderophore piracy in order to uptake enough iron to survive (D'Onofrio et al., 2010).

This presents the possibility of broad-spectrum effects for a siderophore-chelated antibiotic agent.

Specifically, chelating toxic metallic ions to a siderophore creates a complex that is able to infiltrate the cytoplasm of bacteria like a "Trojan Horse", reducing a bacterium's iron intake and increasing the concentration of toxic ions to lead to cell death. Gallium has similar binding properties and atomic radius as iron, so it is ideal for selection as a "Trojan Horse" metal (Minandri et al., 2014). Several studies show that gallium is an effective antibacterial compound (Banin et al., 2008; Olakanmi et al., 2013; Minandri et al., 2014). Chelating gallium to a siderophore complex would allow greater uptake of the ion through natural iron-uptake pathways. Gallium-siderophore complexes also lead to additional toxicity because they compete with normal siderophores for entry into the cell, reducing the amount of iron that can be uptaken. Once inside, gallium also perturbs iron metabolism and generates oxidative stress, leading to cell death (Minadri et. al, 2014). This strategy presents possible advantages over traditional antibiotics.

Existing resistance to conventional antibiotics does not compromise the activity of Ga(III) (Minandri et al., 2014). Therefore, the treatment is likely to be effective even against bacteria that may have already developed resistance to other antibiotics. Drug-resistant *E. coli* synthesize extended-spectrum beta-lactamase, an enzyme that hydrolyzes beta-lactam antibiotics and confers resistance to all penicillins, third-generation cephalosporins, and aztreonam (Shaikh et al., 2015). This enzyme is encoded on a plasmid called CTX-M, that can be transferred horizontally, spreading resistance between different bacterial population (Shaikh et al., 2015). A mono-atomic antibiotic such as gallium (Ga(III)) is not susceptible to the same type of enzymatic

activity as traditional antibiotics, and thus offers another level of protection against the development of resistance.

The efficacy of such a treatment has been evaluated for several species of bacteria in an iron-poor environment (Ahmed et al., 2015). However, current investigations have not determined whether Ga-DFO has broad spectrum treatment effects in non-iron-depleted environments. Therefore, our experiments will investigate the effectiveness of Ga-DFO against a broad spectrum of bacteria in a non-iron-depleted environment as well as in mammalian cell culture. Specifically, we will find an estimate of the minimum inhibitory concentration (MIC), or approximate lowest concentration of Ga-DFO that inhibits visible growth of each bacteria after overnight incubation. In order to fully understand the treatment potential of a Ga-DFO complex, this study will address the following research questions:

Research Questions

1. Does conjugation with siderophore desferrioxamine (DFO) enhance the antibacterial activity of heavy metal ions such as Ga(III)?
2. What is the MIC estimate of Ga-DFO against a broad spectrum of bacteria?
3. Is the MIC estimate equally effective when added during different stages of bacterial growth?
    a. Does the MIC estimate remain effective at inhibiting bacterial growth over an extended time period?
4. Is Ga-DFO's effect bacteriostatic or bactericidal?
5. Does Ga-DFO have an impact on growth rates of mammalian cells?

The answers to these research questions will serve as a foundation for the future development of Ga-DFO as an alternative antimicrobial treatment that does not lead to antibiotic resistance.

We designed three phases of research to answer our research questions and to begin determining the viability of Ga-DFO as a commercially applicable antimicrobial.

Hypotheses

In the first stage of our research, we propose that chelation to siderophores enhance the antibacterial activity Ga(III). We also propose Ga-DFO will be effective against an even broader spectrum of bacteria than has previously been studied because bacteria often uptake foreign siderophores through siderophore piracy (Traxler et al., 2012). This mechanism can allow Ga-DFO to enter the cytoplasms of bacteria that are not natural producers of Ga-DFO.

In the second stage of our research, we propose that Ga-DFO will be effective against bacteria during any stage of bacterial growth. We also propose that Ga-DFO will retain its antibacterial properties in cultures of previously treated bacteria reinfected after a period of two weeks. We chose to investigate reinfection after two weeks because it approximates the long term effects of an antibacterial treatment and allows any metabolic processes involving Ga-DFO (if they exist) to proceed to completion. Bacteria exposed to antibiotics often undergo autolysis (Lewis, 2000), so we postulate that dead bacteria will release cytoplasmic Ga-DFO back into the environment, to be uptaken by other cells. Through this mechanism, Ga-DFO in media should continue to be effective even after being used to treat bacterial cells in a previous contamination.

We propose that Ga-DFO will not cause a significant decrease in mammalian cell growth, and we will investigate this claim in the third phase of the project. Current literature indicates that siderophores are only secreted by microorganisms such as bacteria and fungi (Winkelmann,

1991). Mammalian cells primarily use proteins such as transferrin and ferritin, not DFO, to transport iron (Correnti & Strong, 2012). As a result, we postulate that mammalian cells lack receptors to uptake DFO, and will not be significantly affected by an application of Ga-DFO.

Literature Review

Iron-Uptake as a Target for Antibacterial Treatments

Bacterial cells utilize iron as an enzymatic cofactor for DNA synthesis and repair, metabolic respiration, and oxidative stress response. Iron sources are transported into the Gram-negative bacterial cell through specific uptake pathways, which consists of an outer membrane receptor, a periplasmic binding protein, and an inner membrane ATP-binding cassette transporter. Under physiological conditions, iron can exist in the reduced ferrous (Fe(II)) form or the oxidized ferric (Fe(III)) form, making it extremely versatile for incorporation as catalytic centers or electron carriers in protein (Krewulak & Vogel, 2008). As such, cells cannot function in an iron deficient environment. Since iron uptake is a crucial mechanism to bacterial survival, these defined iron uptake routes common to all bacteria could be utilized to bypass the barrier of the cell membrane (Rabsch, Voigt, Reissbrodt, Tsolis, & Bäumler, 1999) and introduce toxic metals to the cell, acting as an antibacterial treatment.

Siderophore Complexes

Siderophores are small iron-scavenging molecules that are secreted by fungi and bacteria growing in low-iron environments and uptaken after they have formed a complex with iron (Winkelmann, 1991). In bacteria, there are two methods of iron uptake: direct contact between the cell and a Fe(III)/heme source (i.e. receptor-mediated iron acquisition from host proteins) or siderophore-mediated uptake, the latter of which is the focus of the present study. The main difference between these two methods is that heme and siderophores can be taken up by the bacterial cell as intact molecules, while iron must be extracted from host carrier proteins before being transported into the cell (Krewulak & Vogel, 2008). Siderophore complexes show significant promise for use as an antibacterial treatment because many bacteria produce and uptake siderophores.

Although they lack antibacterial properties themselves, siderophores serve as one of the few transport mechanisms capable of penetrating the cell membrane to deliver toxins into the cytoplasm of bacterial cells (Zurenko, Truesdell, Yagi, Mourey, & Laborde, 1990). This capability occurs because bacteria possess multiple siderophore-specific receptors that allow siderophores and their bound substrates to enter and bypass the bacteria's resistance mechanisms (Rabsch, et al., 1999). Moreover, siderophores are capable of entering bacteria other than the ones from which they originated, allowing even artificially constructed siderophores to have a wide range of targets (Traxler et al., 2012). Since the same siderophore could be uptaken by a wide variety of bacteria, a single variety of siderophore complex could act as a broad-spectrum antibacterial treatment. This characteristic of the treatment attributes to its viability as a potential biopharmaceutical product since a single siderophore complex could be used to treat a variety of different infections.

A variety of compounds have been considered to address the problem of growing antibiotic resistance, such as novel antibiotics and heavy metals. Antibacterial treatments that manipulate these routes to bypass the membrane could be highly effective at inhibiting bacterial growth. One area of inquiry has investigated siderophores as a trans-membrane shuttle for current antibiotic treatments. Although iron-chelating complexes can help antibiotics penetrate into the cell, previous research suggests that some of these complexes fail to inhibit bacterial cell growth. Antibiotic-siderophore complexes often fail because once an antibiotic binds to the siderophore, it loses its potency and becomes ineffective as an antibiotic (Page, Dantier, & Desarbre, 2010). Since antibiotic-siderophore complexes are not a feasible antibacterial treatment, alternative applications of siderophores, such as a metal-siderophore complexes, can be considered. Specifically, one potential avenue of treatment would be to induce bacteria to uptake a metal similar in size to iron but with an associated toxicity.

Selecting a Metal for Use in Siderophore Complexes

A complex with a siderophore and a toxic iron-substituting metal would create a treatment that simultaneously deprives bacteria of iron and shuttles toxic ions through the membrane. However, an appropriate metal must be selected to create a siderophore complex that will be toxic to bacteria while avoiding unintentional toxicity to mammalian cells.

Silver is a potential metal for use in a siderophore complex. It has been used as a medicinal metal since the 1800's. It has been applied successfully in eye drops to prevent ophthalmia neonatorum (neonatal conjunctivitis) in infants (Matejcek & Goldman, 2013), in foil form, and as colloidal silver and silver nitrates for burn treatment (Alexander, 2009). The previous success of medical silver suggests it has additional potential in an antibacterial setting. Silver's medicinal benefits do not come without disadvantages. When administered orally, silver can occasionally cause gastrointestinal problems (Alexander, 2009). When administered intravenously, silver can cause convulsions or, even in severe cases, death (Alexander, 2009). The potential of death is severe enough to discount the use of silver as a potential metal for use in the siderophore complex. As such, it is not an ideal candidate for a treatment that could be administered in the body and was thus excluded from this study.

Zinc has also been considered for an antibacterial agent because of its sheer abundance within certain areas of the human body (Ma, Darmawan, Zhang, Zhang, & Bryers, 2013). Since it is naturally plentiful in the human body, it may not cause unintended toxicity to mammalian cells. However, zinc-complexes must be used at exceedingly high concentrations to effectively treat infections, to the point that it becomes toxic to the mammalian host (Ma et al., 2013). When zinc was complexed to protoporphyrin IX, it did not inhibit the growth of either *S. epidermidis* or *P. aeruginosa*. However, when zinc was complex to mesoprotoporphyrin IX, inhibition of bacterial growth could only be achieved at high concentrations. While it is possible to use zinc to inhibit

bacterial growth, the authors demonstrate that a 12.5x less concentrated treatment of gallium can be used to attain the same effect. As such, using zinc is not the most efficient metal to use for antibacterial purposes.

Gallium is an ideal candidate for this use because of its considerable similarity to iron. Ga(III)'s atomic radius and binding properties are nearly identical to those of Fe(III) (Ross-Gillespie, Weigert, Brown & Kümmerli, 2014), and biological systems have difficulty differentiating between the two ions (Ma et al., 2013). As such, gallium can bind easily to iron-scavenging siderophores and cross the cellular membrane through uptake pathways intended for iron. Additionally, Ga(III) is not able to replace Fe(III) in essential redox-dependent reactions in bacteria because Fe(III) reduces readily to Fe(II), but Ga(III) does not reduce to Ga(II) (Ross-Gillespie et al., 2014). Therefore, although iron and gallium are similar, gallium would not be an effective replacement to iron in biological processes, further suggesting its potential for use in an antibacterial treatment.

Siderophore-gallium complexes could introduce toxic gallium ions into bacteria instead of the necessary supply of iron. *In vitro* and *in vivo* data on Ga(III) suggests that some Ga(III) formulations could represent an alternative to conventional antimicrobials for treatment of bacterial infections due to their toxic effect on bacteria. For example, infections caused by some strains of *P. aeruginosa* and *Acinetobacter baumannii* are extremely resistant to currently available antibiotic therapies, while both species are very sensitive to Ga(III) *in vitro* as well as *in vivo* (Minandri et al., 2014). In murine models of pulmonary tuberculosis infection, Olakanmi et al. (2013) observed inhibition of *M. tuberculosis* bacterial growth and a significantly increased survival rate in BALB/c mice and SCID mice treated with $Ga(NO_3)_3$ as compared to control treatments of saline and $NaNO_3$. The concentration of gallium administered in this study was determined safe for use in mammalian cells and feasible for future *in vivo* application (Olakanmi et al., 2013). Gallium was also effective in

treating two different experimental animal infections simulating acute lethal pneumonia and a chronic airway biofilm infection (Banin et al., 2008). Therefore, gallium could be an effective method for treating bacterial infections. Repurposing Ga(III) as an antibacterial agent offers an opportunity to take advantage of the established pharmacological properties of Ga(III)-based formulations, thus accelerating clinical testing of its antibacterial properties (Minandri et al., 2014).

Even before entering the cell, gallium competes with iron for siderophore binding sites, thereby limiting the supply of iron available to be uptaken via siderophores (Ross-Gillespie et al., 2014). While gallium is effective as an antimicrobial agent alone, linking it to a siderophore and encouraging greater uptake through manipulation of natural iron-uptake pathways could create a treatment that is even more effective in inhibiting bacterial growth. Pathogens such as *Pseudomonas aeruginosa*, *Francisella tularensis*, *Mycobacterium tuberculosis*, and *E. coli* are sensitive to iron starvation (Rzhepishevska et al., 2014), so inducing bacteria to uptake a different metal could starve the cell of essential iron while introducing toxic ions into the cytoplasm of the bacterial cell. While a toxic ion-siderophore complex would not necessarily completely prevent iron uptake, any mutations that allow the prevention of uptake of the toxic siderophore would also prevent the uptake of essential iron. Therefore, it could be less likely that bacteria can easily adapt to avoid uptake of the toxic ions.

After considering all possible metals for use in a metal-siderophore complex, gallium was the most appropriate candidate because of its similarity to iron in atomic size and binding properties, as well as its previous use as an antibiotic. When implementing a gallium treatment, one must use a concentration that is effective at inhibiting bacterial growth without unintentionally harming surrounding cells. Too high of a concentration may cause gallium to travel across the cell membrane and interfere with iron metabolism in the mammalian cell culture where treatment is being applied, causing toxicity to these cells (Ross-Gillespie et al., 2014). When compared to free gallium, smaller

concentrations of a gallium-siderophore complex are necessary for treatment since it penetrates the cell membrane more effectively through natural iron uptake pathways. Gallium-siderophore complexes thus show promise in retaining effectiveness at a concentration that will not harm mammalian cells.

Previous Research on Gallium-Siderophore Complexes

Previous research has examined metal-siderophore complexes such as Ga-DFO, Ga-cit (citrate), Ga-Staphyloferrin A, Ga-PPIX (protoporphyrin IX), Ga–pyoverdine, and Ga–pyochelin. Cepaciachelin and dihydroxybenzoyl-serine, two catechol-based siderophores produced by Gram-negative bacteria, were ruled out for this study after previous research determined that they were no more effective than a multitude of simpler, more available siderophores (Kelson et al., 2013). The heterologous siderophore DFO is a potential candidate for complex with gallium because of its commercial availability and past research on its properties. Specifically, Banin et al. (2008) demonstrated that a complex between Ga(III) and DFO was slightly more effective than Ga(III) alone in killing *P. aeruginosa* cells, both in established biofilms and *in vivo*. It is important to note that while that study demonstrated the effective bactericidal properties of Ga-DFO, it did not investigate the properties of Ga-DFO with regard to bacterial specificity and robustness against resistance. It also investigated the effects of Ga-DFO in a low iron medium, which is less clinically and commercially relevant than an iron-sufficient environment.

Compared to Ga-DFO, Ga-cit exhibits broad activity against many Gram-negative bacteria at ~1–5 μg/ml MICs, as well as strong biofilm activity, low drug resistance, and efficacy *in vivo* resulting in higher uptake of gallium by *P. aeruginosa* (Kelson et al., 2013). Not only that, but the Ga–citrate complex also showed higher bacteriostatic and bactericidal activities than Ga-DFO and was more effective in protecting macrophages from infection, plausibly due to a more efficient

uptake of Ga–citrate by *P. aeruginosa* cells (Minandri et al., 2014). However, Ga–cit was found to exert a negligible inhibitory activity on a number of other species, including *E. coli* (Minandri et al., 2014). Citrate-mediated active uptake of Ga(III) may not be sufficient for Ga(III) toxicity against a broad spectrum of bacteria. Additionally, with use of Ga-cit, some precipitation can occur at pH levels above 7 (Rzhepishevska et al., 2011). Due to these two faults, Ga-cit is not an ideal candidate for a broad spectrum antibacterial treatment that is effective in many environmental conditions.

Staphyloccocus aureus strains such as methicillin resistant strains (MRSA) produce the natural siderophore, staphyloferrin. When investigating staphyloferrin as a siderophore component, little antibacterial activity was detected, regardless of the metal conjugate, indicating that staphyloferrin is not ideal for use in an antibacterial complex (Kelson et al., 2013). Previous research indicates that because the siderophore staphyloferrin is native to *Staphylococcus aureus,* MRSA may have the ability to distinguish between Ga-staphyloferrin and Fe-staphyloferrin. Thus, creating a gallium complex with a native siderophore may result in poor antimicrobial activity (Kelson et al., 2013). Bacteria are able to produce their own siderophores and utilize the siderophores produced by other bacteria. Desferrioxamine, the native siderophore for *Streptomyces pilosus*, would therefore be a viable option for the purpose of our experiment, since no strains from the Streptomyces genus will be studied (Bergeron & Brittenham, 1994). This way, the strains being studied will be less likely to differentiate between the iron and gallium complexes.

Research suggests that Ga-PPIX (protoporphyrin IX chloride) is endowed with strong antibacterial activity against a wide range of Gram-positive and Gram-negative bacterial pathogens (Minandri et al., 2014). Among the different bacteria samples treated with Ga-PPIX, *Bacillus subtilis, E. coli, Helicobacter pylori, Listeria monocytogenes, Mycobacterium bovis, Neisseria gonorrhoeae, Neisseria meningitidis, Yersinia enterocolitica,* and *Yersinia pseudotuberculosis* were the most

sensitive [MICs ≤ 0.5 μg/ml, corresponding to 0.8 μM Ga(III)]. Yet certain characteristics associated with this siderophore complex display its limited efficacy. For instance, Ga-PPIX was most active against bacterial strains grown in an environment with medium or high concentrations of iron (Stojiljkovic et al., 1999). In addition, other drawbacks involving bacteria that don't express haem uptake systems or contain cytochromes were shown to significantly reduce the level of antibacterial activity.

Gram-negative species lacking heme uptake systems, such as *Salmonella Typhimurium*, and microorganisms that do not contain cytochromes, such as facultative anaerobes, were found to be resistant to Ga-PPIX (Minandri et al., 2014). Moreover, Ga-PPIX showed relatively low aqueous solubility, modest toxicity, and a complex synthetic scheme (Kelson et al., 2013). Obligate and facultative anaerobic bacteria using fermentation for energy production were found to be resistant to Ga-PPIX (Minandri et al., 2014). Ga-pyoverdine and Ga-pyochelin complexes are recognized and actively internalized by cognate outer membrane receptors; however, the inhibitory activity of Ga(III) on *P. aeruginosa* was not influenced by the presence of these siderophores and their uptake systems (Minandri et al., 2014). The fact that bacteria have already developed resistance to these complexes renders them unviable for use in clinical settings.

After comparison of many possible complexes for use as an antibacterial treatment, we determined that Ga-DFO is the most suitable metal-siderophore complex because Ga-DFO presents the possibility for broad spectrum effects via siderophore piracy (Traxler, Seyedsayamdost, Clardy, & Kolter, 2012), it is easy to acquire, and previous studies have suggested its efficacy as an antibacterial agent *in vivo*. When Ga-DFO was applied to *P. aeruginosa*, it inhibited growth, prevented biofilm formation, and showed bactericidal activity against free-living as well as biofilm cells (Banin et al., 2008). Additionally, Ga-DFO treatments at a concentration of 5.15 mM served to significantly

reduce bacterial growth rates when compared to a treatment of gallium ions alone for four strains of bacteria (Ahmed et al., 2015). Intriguingly, Ma et al. (2013) found sub-inhibitory concentration of Ga(III) and Ga-DFO (1 μM) can prevent biofilm formation without killing any mammalian cells. The finding that treatment with Ga(III) alone can inhibit growth questions validity of the "Trojan Horse" model. Thus, further research should be conducted to examine the difference in impact between gallium and Ga-DFO treatments.

Potential Applications

One potential application for Ga-DFO could be a surface treatment on abiotic surfaces and objects. A goal of this project is to contribute to the development of a novel abiotic antibacterial treatment. In particular, rates of antibiotic-resistant *E.coli* infection are rapidly rising worldwide. Traditional treatments such as fluoroquinolones and third- or fourth- generation cephalosporins are rendered ineffective by the rise of a particular extended spectrum beta-lactamase (ESBL) strain of *E. coli* (Collignon, 2009). A new class of antibiotics such as Ga-DFO present a possible solution for treatment of food-borne infections of *E. coli.*

Ventilator-associated pneumonia is a common nosocomial infection that significantly contributes to morbidity in intensive care unit patients. Catheter-associated urinary tract infection (CAUTI) is another common nosocomial infection which is often caused by the formation of biofilm on catheter surfaces. He et al. (2012) found that treatment with an antimicrobial spray, called JUC, significantly reduced the incidence of CAUTI in hospital patients. JUC spray inhibits the growth of microorganisms by creating a positively charged film on the target surface. Addition of Ga-DFO to such a treatment could increase the potency of this treatment by adding bactericidal/bacteriostatic properties. Pending further research and development, Ga-DFO also presents possibilities for greater application against other bacterial infections as well, since DFO is utilized by a variety of obligate

and facultative anaerobic microbes (Neilands, 1995). Antimicrobial sprays involving modified siderophores could be a novel way to prevent nosocomial infections without contributing to antibiotic resistance, especially considering the necessity for iron in biofilm formation (Banin et al., 2005).

As antibiotic resistance continues to persist and progress, new methods of combating bacteria must be developed. Gallium-siderophore complexes are a water-soluble compound with potential for development into a spray. In this study, we examine the antibacterial activity of Ga-DFO against a variety of bacteria, as well as its antibacterial properties in mammalian culture.

Methodology

Methodology Overview

In order to answer our research questions, we conducted our project in three phases. In Phase I of our project, we examined our first and second research questions. We conducted a series of experiments examining the effect of Ga-DFO treatment on various bacterial cell cultures including *E. coli, Enterobacter aerogenes, S. aureus*, and *S. typhimurium*. For the purposes of our research, we defined "treatment" as the administration of a predetermined concentration of Ga-DFO into a bacterial culture. We report the MIC estimate of Ga-DFO for a selection of motile bacterial cultures in a non-iron-depleted environment.

Phase II of our research experiment investigated the properties of Ga-DFO as a compound and antibacterial treatment in order to answer research questions three and four. We investigated the effectiveness of Ga-DFO treatment at different stages of bacterial growth and

determined whether the Ga-DFO treatment had a bacteriostatic or bactericidal effect using CFU analysis. We also determined whether Ga-DFO maintains an antibacterial effect over time by reinfecting a treated plate with *E. coli* after two weeks.

Phase III of our research examined the applicability of Ga-DFO in mammalian cell cultures and addressed the fifth research question. We used Suspension-adapted Freestyle Chinese Hamster Ovary (CHO-S) cells and Fibercell Human Embryonic Kidney (HEK) adherent cells. We treated non-contaminated cultures of CHO-S cells and HEK cells with applications of Ga-DFO and determined if it caused a decrease in the CHO-S cells' growth rate. If the treatment was found to have no effect on CHO-S cells or HEK cells' growth rate, we planned to determine if Ga-DFO is still effective at treating bacteria in CHO-S and HEK cell culture.

Bacterial Selection

We selected bacterial strains based on their clinical relevance and whether or not they produced siderophores. The selected bacteria differ in biosafety levels (BSL**)** and gram type. Gram-positive bacterial resistance to antibiotics is increasing, which is responsible for many infections that affect patients in hospitals (Rice, 2006). Gram-negative bacteria are more resistant because the outer membrane provides a barrier for antibiotic entrance into the cell (Delcour, 2008).

Bacterium	**BSL**	**Gram Type**	**Siderophore Production/ Receptor**	**Clinical Relevance**	**ATCC Code**

Enterobacter aerogenes	1	-	Typically produces the enterobactin siderophore and some isolates may produce aerobactin (Mokracka, Koczura, & Kaznowski, 2004)	Rarely causes disease in healthy individuals, but can affect immunocompromised patients; historically associated with antibiotic resistance (Sanders & Sanders, 1997)	29007
Escherichia coli	1	-	Most strains of *E. coli* produce the enterobactin siderophore, but certain isolates also produce aerobactin (Jacobsen et al., 2008); Able to use siderophores produced by other species as well (Neilands, 1995)	Common cause of catheter-associated urinary tract infections (CAUTI), which are the most common nosocomial infections; Present in 70—90% of community acquired UTI's (Jacobsen et al., 2008)	11303
Salmonella Typhimurium	2	-	Produces enterobactin and salmochelin siderophores (Crouch, Castor, Karlinsey, Kalhorn, & Fang, 2008)	Associated with foodborne illness; known to be multi-drug resistant (Helms, Vastrup, Gerner-Smidt, & Mølbak, 2002)	13311

Staphylococcus aureus	2	+	Production of staphyloferrin A, staphyloferrin B, staphylobactin, and aureochelin siderophores; Hydroxamate siderophore receptor (Dale, Doherty-Kirby, Lajoie, & Heinrichs, 2004)	Associated with conditions such as skin infections, toxic shock syndrome, and food poisoning (Schlievert et al., 2000)	12600

Table 1. Characteristics of bacterium used.

Data Collection

Preparation of Bacterial Cell Culture. All bacterial cell lines were kindly provided by the Microbiology Undergraduate Teaching department at the University of Maryland. For all experiments, suspension cultures of bacteria were grown in Luria broth [LB] (10g Tryptone, 10g NaCl, 5g Yeast Extract per 1L of diH_2O) at 37℃. All bacteria were grown to mid-log phase for each experiment. We define mid-log as the period in cell culture when bacteria experience exponential growth, as evidenced by a sharp increase in optical density readings (OD). Optical density readings were measured at 600 nm wavelength.

Ga(III)/Ga-DFO Treatment Preparation. Ga-DFO can be synthesized in solution with diH_2O as a solvent. Equimolar amounts of DFO-mesylate salt (Glentham Life Sciences, Product #GP5073) and $Ga(Cl)_3$ (Alfa Aesar, Catalog Number AA43879-06) were added to diH_2O, then added to an appropriate concentration of LB. We used an adapted chrome azurol S (CAS) assay (Ahmed et al., 2015) to confirm complexation.

CAS assay is a test that utilizes the molecule chrome azurol S (CAS) to detect the presence of siderophores in a cell culture. The molecule CAS forms a ternary complex with Fe(III) and a molecule known as hexadecyltrimethylammonium bromide (HDTMA).

Siderophores compete with the dye to chelate Fe(III). This can be further expressed through the following chemical equation:

$$FeDye^{3-\lambda} + L^{\chi-} \rightarrow FeL^{3-\chi} + Dye^{\lambda-}$$

The ternary complex is represented by *FeDye* and the siderophore is represented by the *L* symbol. There is a clear indication that the reaction has occurred when the dye color changes from blue to red once the iron has detached from the CAS complex and bonded to the siderophore. In our experiment, we used desferrioxamine B (DFO-B) as our siderophore and gallium as our metallic ion, but the same principles apply because gallium has many of the same properties as iron (Ross-Gillespie et al., 2014). We followed the protocol for the CAS Assay as referenced in **Appendix A1** (Ahmed et al., 2015).

Phase I: MIC estimate of Ga-DFO

We compared the antibacterial activity of a Ga(III) treatment with a Ga-DFO treatment via OD readings over a 15 hour microplate fermentation. Both treatments were administered at either 0.005M or 0.05M concentrations. After observing our results (Figures 1-4), we proceeded to determine the MIC estimate of Ga-DFO against *E. coli*. We define MIC estimate as an approximation of the lowest concentration at which Ga-DFO inhibited the logarithmic growth of a given culture of bacteria over a 15 hour microplate fermentation.

Microplate Fermentation. We used microplate fermentation to determine the effect of treatment compounds for each experiment. For microplate fermentation, mid-log cultures of *E. coli, S. typhimurium, S. aureus* and *E. aerogenes* were diluted to OD600=0.3 and then inoculated at a 1:25 ratio with LB into a 24 well plate (final volume 2.5 mL), then incubated inside a Biotek Synergy HT multi-mode microplate reader (shake speed slow, 37℃, 15 min read intervals,

OD600nm). We used the protocol in **Appendix A2.1** for microplate preparation and data collection.

For the purposes of this study, "experiment" refers to one set of microplate fermentations performed simultaneously in the same microplate with the same species of bacteria under the same treatment. The term "trial" means a single fermentation in a single well of a microplate.

Phase I controls. To account for possible confounding variables in the results of our data, we compared the effect of the Ga-DFO treatment to an no treatment sample and two control treatments. The first control was a treatment of free gallium ions at the same concentration as the Ga-DFO treatment. The second control treatment was *E. coli* grown without treatment. This served as a point of comparison to examine the effects of the DFO- shuttle mechanism for gallium delivery.

In Phase I, we tested the Ga-DFO treatment on *E. coli, E. aerogenes, S. aureus*, and *S. typhimurium*. However, we focused on *E. coli* for subsequent experiments because it is used as a model for bacteria of its simplicity, doubling time and ability to grow with different nutrient mixtures. It is a single-celled organism that is easy to grow and manipulate. *E. coli* has a doubling time of approximately 20 minutes so it can be reproduced in experiments quickly. The small size of its genome provides advantages and its entire genetic sequence has been determined. The bacterium grows most rapidly in mixtures that include glucose, salts and organic compounds, but it is also able to grow in minimal media that consists of salts, a nitrogen source and a carbon source (Cooper, 2000).

Phase II: Properties of Ga-DFO

CFU Analysis. In order to assess bacterial cell viability after treatment, we performed cell density analysis by counting colony forming units. This provided a metric to assess the bacteriostatic or bactericidal activity of Ga-DFO. *E. coli* was grown to mid-log phase then treated with an equal volume of 0.100 M Ga-DFO LB (final concentration 0.05 M Ga-DFO). CFU counts were taken at 5 min, 10 min, and 30 min after treatment using minicolony techniques (Sieuwarts et al., 2008). Colony forming units were counted after 18 hours incubation at 37℃. From our findings, we proceeded to use 0.05 M Ga-DFO in subsequent experiments. Further information on this experiment can be found in the **Appendix 3**, and the results are presented in Figure 6.

Time-Dependent Dosage. To assess Ga-DFO's efficacy against bacteria throughout various growth phases, a time dosage experiment was conducted. For this experiment, *E. coli* was grown using microplate procedures, and treated with Ga-DFO at 1, 4, 8 and 16, and 24 hours after bacterial inoculation. *E. coli* reaches mid-log phase in approximately 6 hours, so treatment at various time points before, during, and after this stage will reveal the potency of Ga-DFO against *E. coli* in various stages of growth. All treatments were performed in quadruplicate. This is important to understanding the functionality of Ga-DFO in real world applications. Further information on this experiment is available in **Appendix 4**, and the results are presented in Figure 7.

Two Week Media Transfer An opportunity was also available to adapt the time dosage experiment to test whether Ga-DFO has bactericidal or bacteriostatic effect. Upon completion of the time dosage experiment, the 24 well plate was incubated at 37℃ for two weeks. Prior to re-exposure, cell viability was measured using mini-colony CFU analysis. Afterwards, duplicates of the two week old culture were supplemented with new LB media and incubated using microplate

fermentation. Cell viability was measured again with microplate fermentation. Growth of these cultures would show that viable bacteria is still present, indicating that Ga-DFO has a bacteriostatic effect and simply stops cell proliferation. No growth would indicate that Ga-DFO has a bactericidal effect and kills cells completely. Further information on this experiment is available in **Appendix 5**, and the results are presented in Figure 8.

Two Week Reinfection. The other duplicates of the wells received an equal volume of new LB media (final [Ga-DFO]= 0.025M), and mid-log *E. coli* diluted to OD=0.3. The plate was incubated once more using microplate fermentation. One possible bacterial defense to any antibiotic is degradation of the antibiotic. A week-long incubation period allowed Ga-DFO in culture to fully react with any possible metabolic enzymes, so that we can also assess the potential longevity of the treatment in a practical application. Further information on this experiment can be found in **Appendix 5**, and the results are presented in Figure 9.

Phase II controls. In order to ensure no contamination was present and growth inhibition was as expected, the following controls were implemented. All measurements of cell growth or cell viability were taken alongside a "no treatment" sample. For any microplate fermentation, a quadruplicate of wells with only LB media was used as a check for contamination in the plate, and as a reference for no growth. In addition, a quadruplicate of wells with LB media and *E. coli* was used as a reference for untreated growth.

Phase III: Ga-DFO in Mammalian Cell Culture

Prior research suggests safe interaction between Ga-DFO and mammalian cells, which is crucial for future commercial applications of Ga-DFO but has not yet been studied sufficiently. In this study, the effects of Ga-DFO were tested against Human Embryonic Kidney (HEK) cells and CHO-S cells to observe the interactions mammalian cells have with Ga-DFO. Based on the

results of phase I, a concentration of 0.005M was used for this phase. This research is intended to allow for future research to focus on experiments specific to practical applications of Ga-DFO.

CHO-S cells were grown in Freestyle Serum Free CHO media + 0.008M L-Glut. HEK cells were grown in DMEM with 10% FBS and 1% Penicillin-Streptomycin + 0.008M L-Glut. Cells were incubated at 37℃, and viability determined using automated ViCell counter (Beckman Coulter) and Trypan Blue staining methods at 1 and 24 hours after inoculation for CHO-S cells and at 24 hours after inoculation for HEK cells.

Phase III controls. To understand the interaction between mammalian cells and Ga-DFO we treated mammalian cells from two different species with Ga-DFO. We also compared the effects of the Ga-DFO treatment to diluted mammalian cell cultures treated with extra media to mimic the decrease in cell density when treatment is added. Cell counts were completed before passage to ensure cells are alive and replicating before treatment or dilution. To ensure sterility and culture integrity, the only non-media fluid used was sterile filtered ultrapure type 1 water.

Statistical Analysis

ANCOVA (one-way analysis of covariance) was used to compare the means of the dependent variable based on the independent variable while controlling for the effects of the continuous variable of time for all OD measurements taken from microplate fermentation. This analysis was carried out for each media condition in order to determine if the differences in cell count between treatment groups were statistically significant. The independent variable was treatment group (i.e. .05 M Ga-DFO, .005 M Ga-DFO, or Control). The dependent variable was optical density, and the covariate variable was time.

Results

Microplate fermentation

A one-way ANCOVA was used to compare the effectiveness of a 0.005M Ga(III) treatment and 0.005M and 0.05M Ga-DFO treatments while controlling for time for each bacterial strain. The response of *E. coli* to our treatment is shown in Figure 1.

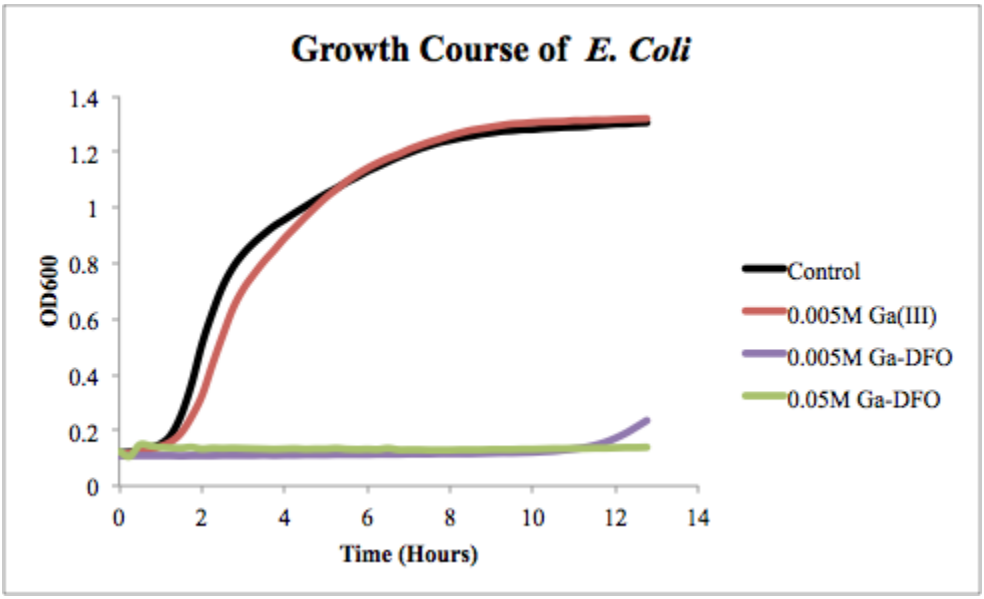

Figure 1. Growth course for *E. coli* after administration of 0.005M gallium, 0.005M Ga-DFO, and 0.05M Ga-DFO treatment. Treatment was added to liquid culture in the lag phase of growth.

Treatment A	[Treatment A], (M)	Treatment B	[Treatment B] (M)	P-value
Ga-DFO	0.05	Ga-DFO	0.005	1.000
Ga-DFO	0.005	Ga	0.005	0.000*
Ga-DFO	0.005	Ga	0.005	0.000*
Ga-DFO	0.05	Control	N/A	0.000*
Ga-DFO	0.005	Control	N/A	0.000*

Table 2. Statistical significance of microplate fermentation of *E. coli* in the presence of Ga(III) and Ga-DFO treatments. Asterisks indicate significant trials.

Gallium treatment alone shows little to no effect on the growth of the bacteria, while 0.005M and 0.05M Ga-DFO lead to almost complete inhibition of growth. Results showed that

there is a significant difference in OD [F(4,51)=253.248, p=0.000] between the treatments of *E. coli*. Post hoc tests showed a significant difference between 0.005M Ga-DFO and 0.005M Ga(III) (p=0.000) and 0.05M Ga-DFO and 0.005M Ga(III) (p=0.000).

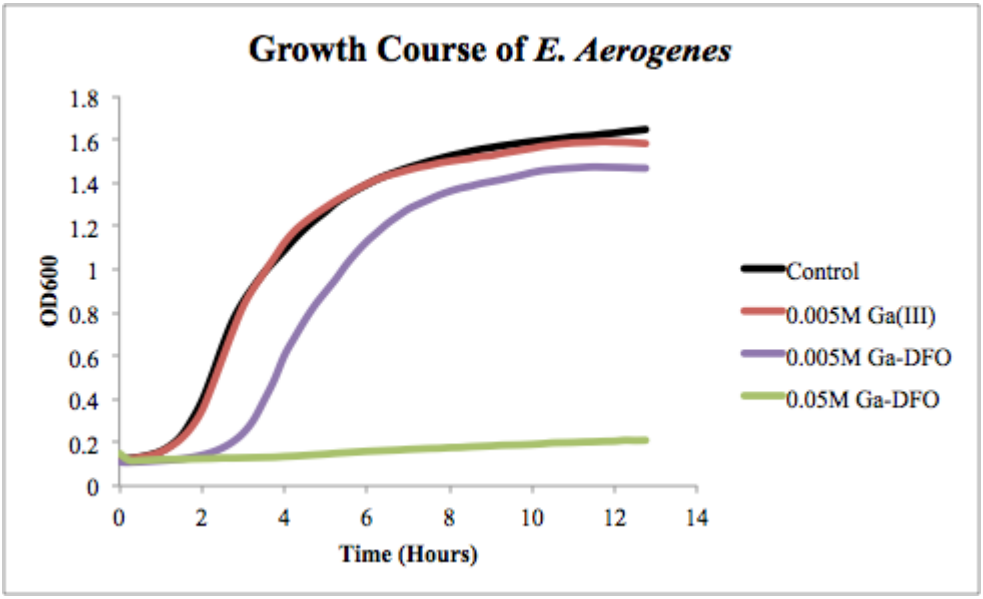

Figure 2. Growth course for *E. aerogenes* after administration of 0.005M gallium, 0.005M Ga-DFO, and 0.05M Ga-DFO treatment. Treatment was added to liquid culture in the lag phase of growth.

Treatment A	**[Treatment A], (M)**	**Treatment B**	**[Treatment B] (M)**	**P-value**
Ga-DFO	0.05	Ga-DFO	0.005	0.000*
Ga-DFO	0.05	Ga	0.005	0.000*
Ga-DFO	0.005	Ga	0.005	0.001*
Ga-DFO	0.05	Control	N/A	0.000*
Ga-DFO	0.005	Control	N/A	0.000*

Table 3. Statistical significance of microplate fermentation of *E. aerogenes* in the presence of Ga(III) and Ga-DFO treatments. Asterisks indicate significant trials.

While the gallium treatment shows little to no effect on the growth of the bacteria, inhibition of growth is observed at a concentration of 0.05M Ga-DFO. A 0.005M Ga-DFO treatment significantly inhibited growth compared with the control and gallium treatment, but

did not completely inhibit all growth. *E. aerogenes* growth showed a significant difference in OD [F(4,51)=189.854, p=0.000] between the treatments. Specifically, there is a significant difference between 0.005M Ga-DFO and 0.005M Ga(III) (p=0.001) and 0.05M Ga-DFO and 0.005M Ga(III) (p=0.000).

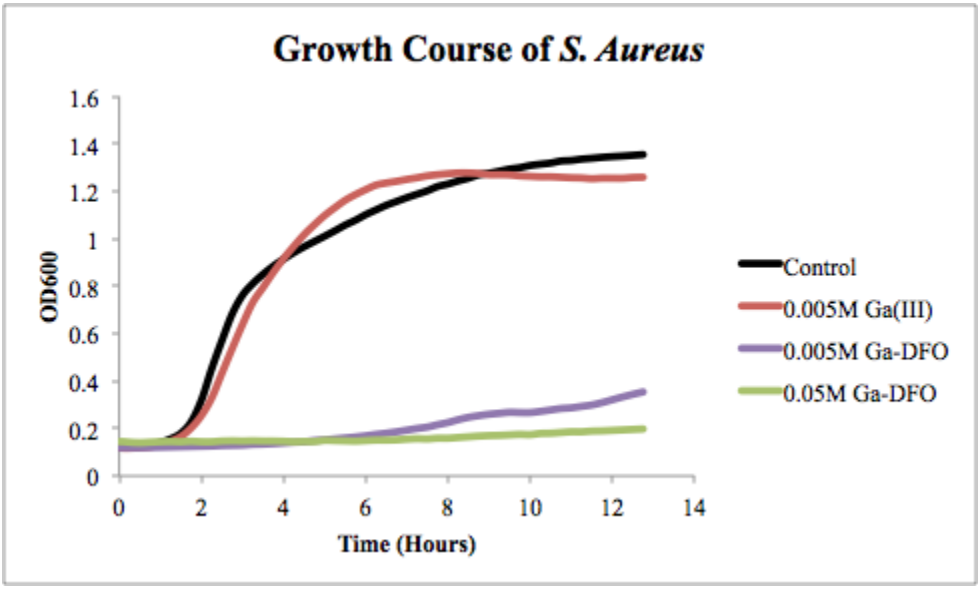

Figure 3. Growth course for *S. aureus* after administration of 0.005M gallium, 0.005M Ga-DFO, and 0.05M Ga-DFO treatment. Treatment was added to liquid culture in the lag phase of growth.

Treatment A	[Treatment A], (M)	Treatment B	[Treatment B] (M)	P-value
Ga-DFO	0.05	Ga-DFO	0.005	1.000
Ga-DFO	0.05	Ga	0.005	0.000*
Ga-DFO	0.005	Ga	0.005	0.000*
Ga-DFO	0.05	Control	N/A	0.000*
Ga-DFO	0.005	Control	N/A	0.000*

Table 4. Statistical significance of microplate fermentation of *S. aureus* in the presence of Ga(III) and Ga-DFO treatments. Asterisks indicate significant trials.

The gallium treatment shows no effect on *S. aureus* growth, but an inhibition of growth is observed at concentrations of 0.005M and 0.05M Ga-DFO. There is a significant difference in OD [F(4,51)=224.132, p=0.000] between the treatments. Post hoc tests show a significant

difference between 0.005M Ga-DFO and 0.005M Ga(III) (p=0.000) and 0.05M Ga-DFO and 0.005M Ga(III) (p=0.000).

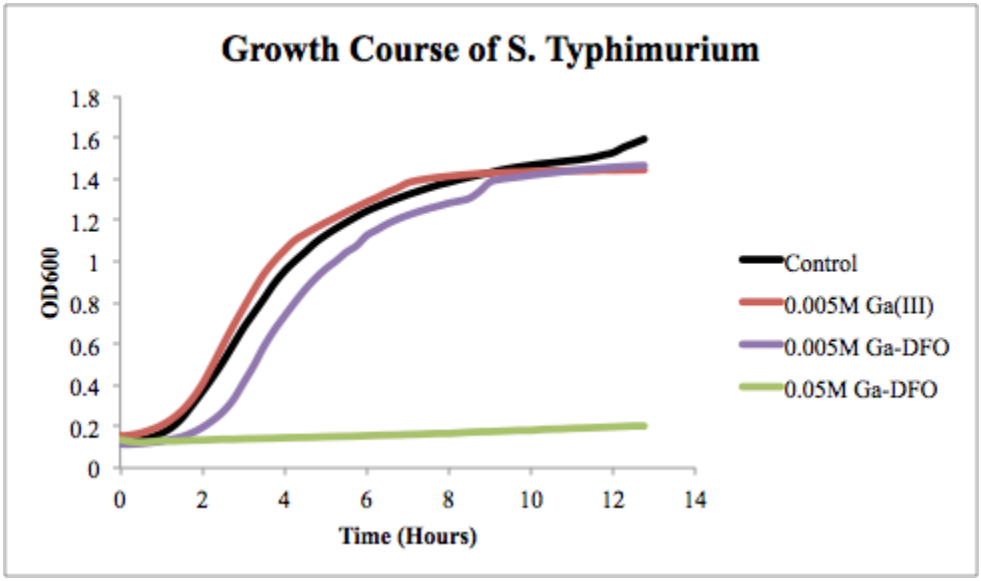

Figure 4. Growth course for *S. typhimurium* after administration of 0.005M gallium, 0.005M Ga-DFO, and 0.05M Ga-DFO treatment. Treatment was added to liquid culture in the lag phase of growth.

Treatment A	**[Treatment A], (M)**	**Treatment B**	**[Treatment B] (M)**	**P-value**
Ga-DFO	0.05	Ga-DFO	0.005	1.000
Ga-DFO	0.05	Ga	0.005	0.000*
Ga-DFO	0.005	Ga	0.005	0.000*
Ga-DFO	0.05	Control	N/A	0.000*
Ga-DFO	0.005	Control	N/A	0.000*

Table 5. Statistical significance of microplate fermentation of *S. typhimurium* in the presence of Ga(III) and Ga-DFO treatments. Asterisks indicate significant trials.

Gallium treatment shows little to no effect on the growth of the *S. Typhimurium*. 0.005M Ga-DFO leads to a slight reduction in bacterial cell count, and 0.05M Ga-DFO leads to a 1-log reduction of final cell count. There is a significant difference in OD [F(4,51)=272.445, p=0.000]

between the treatments. Post hoc tests show a significant difference between 0.005M Ga-DFO and 0.005M Ga(III) (p=0.000) and 0.05M Ga-DFO and 0.005M Ga(III) (p=0.000).

Minimum Inhibitory Concentration of *E. coli*

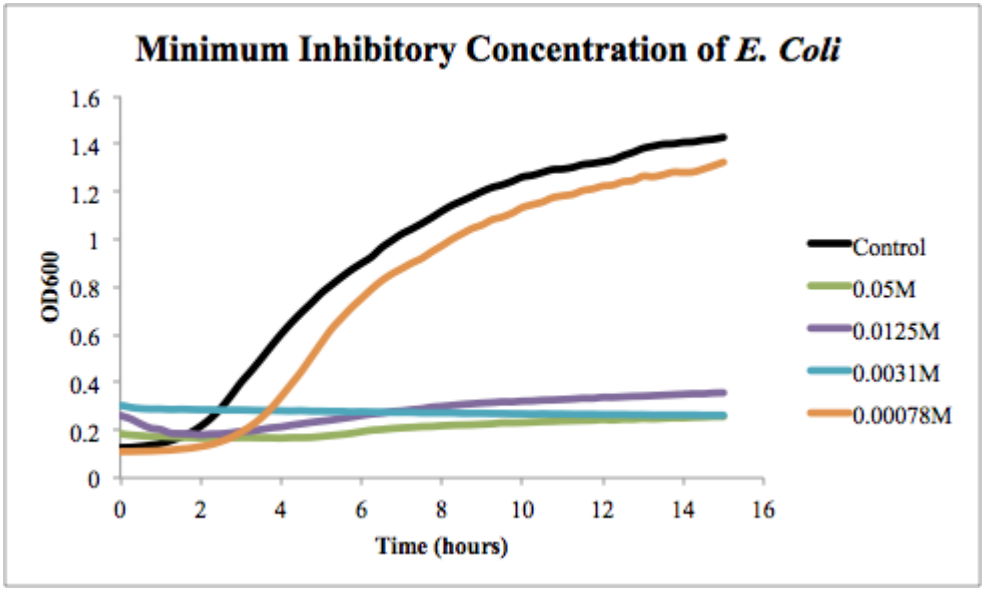

Figure 5. Growth course of *E. coli* after administration of 0.05M, 0.0125M, 0.0031M and 0.0078M Ga-DFO treatment. Treatment was added to liquid culture in the lag phase of growth.

Treatment A	[Treatment A], (M)	Treatment B	[Treatment B] (M)	P-value
Control	N/A	Ga-DFO	0.05	1.000
Control	N/A	Ga-DFO	0.0125	0.000*
Control	N/A	Ga-DFO	0.0031	0.000*
Control	N/A	Ga-DFO	0.00078	0.007*

Table 6. Statistical analysis for MIC estimate tests. P-value indicates statistical significance of OD difference between control and Ga-DFO concentrations. Asterisks indicate significant trials.

Administration of Ga-DFO at a concentration down to 0.0031M completely inhibited growth of *E. coli* in microplate fermentation. We thus determine the MIC of Ga-DFO against *E.*

coli to fall within a range between 0.0031 M and .00078 M. There is a significant difference in OD [F(4, 299)= 146.394, p=0.000] between concentrations. Post hoc tests show a significant difference between all concentrations of Ga-DFO tested, including 0.0031 M to 0.05 M Ga-DFO compared to control (p=0.000) and 0.00078 M Ga-DFO compared to control (0.007). While 0.00078 M Ga-DFO inhibited growth to a statistically significant degree, it did not completely prevent further growth as the higher concentrations did and is therefore below the MIC.

Colony Forming Unit Analysis

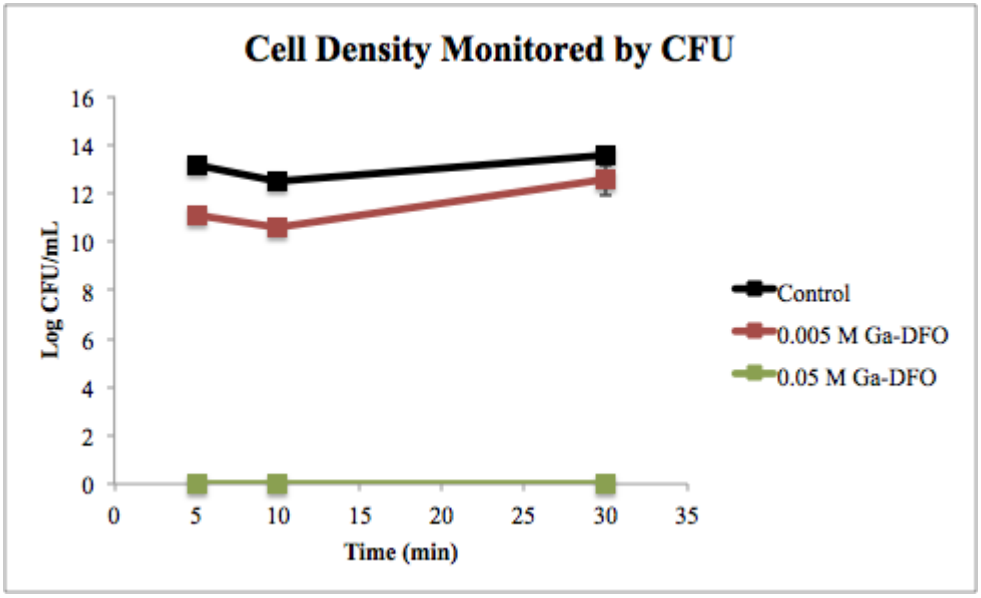

Figure 6. Measured cell density of *E. coli* culture 5 , 10, and 30 min after administration of Ga-DFO. Cell density is presented in logarithmic form (base =10).

Treatment A	[Treatment A], (M)	Treatment B	[Treatment B] (M)	P-value
Control	N/A	Ga-DFO	0.005	1.000
Control	N/A	Ga-DFO	0.05	0.000*
Ga-DFO	0.005	Ga-DFO	0.05	0.027*

Table 7. Statistical analysis for CFU analysis. P-values indicate statistical significance of OD difference between groups. Asterisks indicate significant trials.

CFU analysis showed negligible bactericidal activity when 0.005 M Ga-DFO was administered at mid-log phase, as evidenced by a negligible decrease in colony forming units for all time points after treatment. 0.05M Ga-DFO completely eliminated all colony forming units at 5 min, 10, and 30 min after treatment. There was a significant difference in cell density between groups [F(2, 5)= 11.888, p= 0.013]. Post hoc tests show a significant difference between 0.005M and 0.05M (p=0.027) and control and 0.05M (p=0.000).

Time-Dependent Dosage

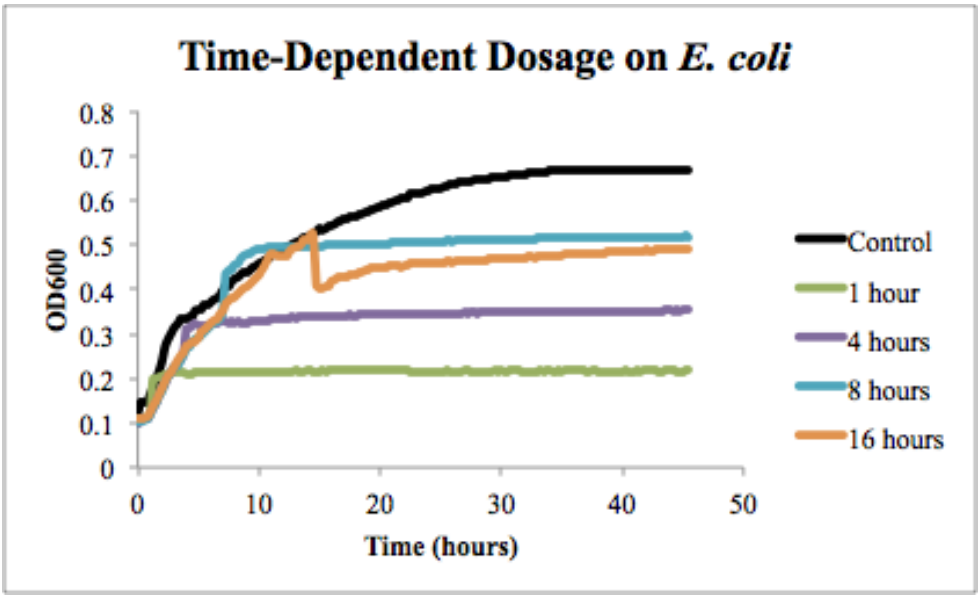

Figure 7. Growth curve for *E. coli* with 0.05M Ga-DFO added at 1, 4, 8, 16 hours and 24 hours during the growth curve compared to *E. coli* alone.

Treatment time	**P-value**
1 hour	0.000*
4 hours	0.000*
8 hours	0.000*
16 hours	0.000*

Table 8. Growth of *E. coli* with 0.05M Ga-DFO added at different points during the growth curve. P-values indicate the statistical significance of differences in OD between the control (*E. coli* alone) and the treated culture after time of treatment. Asterisks indicate significant trials.

Administration of Ga-DFO completely halted growth at its respective time point. Statistical analysis indicates there was a significant difference in OD between *E. coli* alone and *E. coli* with Ga-DFO added at 1 hour [F(1,367)= 2398.092, p=0.000]. There was also a significant difference with Ga-DFO added at 4 hours [F(1,343)= 1900.089, p=0.000], 8 hours [F(1,311)=528.862, p=0.000] and 16 hours [F(1,247)= 6672.418, p=0.000].

Two Week Media Transfer

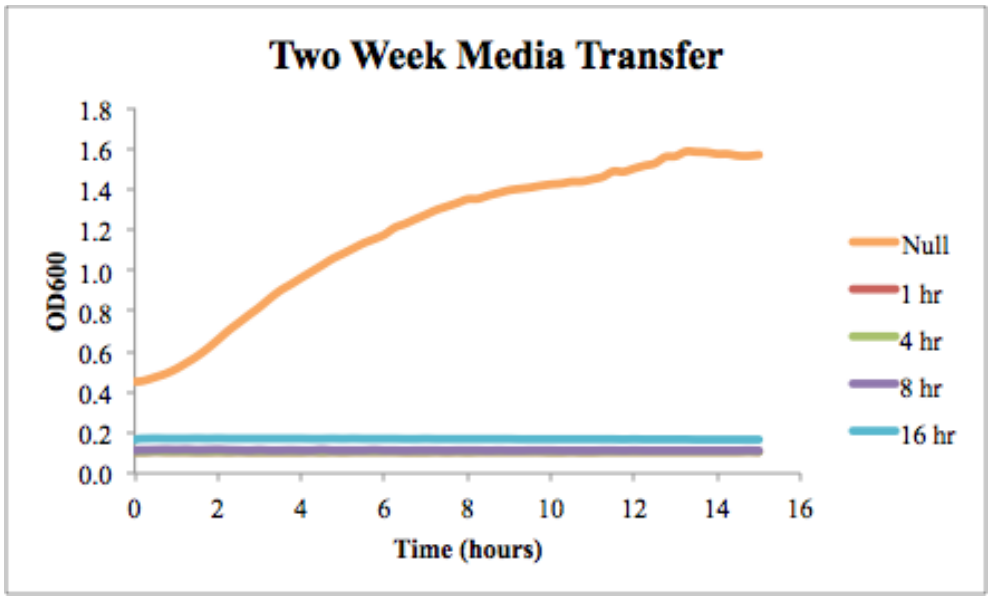

Figure 8. Growth course of two-week old time dosage cultures refreshed with new LB media. Wells previously treated with 0.05M Ga-DFO exhibited no growth, but a previous no treatment well exhibited normal growth.

After 2 weeks of incubation, CFU analysis showed 0 observable colonies. 1, 4, 8 and 16 hour treatment wells did not exhibit growth after refreshment with new LB media. In contrast, 2-week old *E. coli* with no drug treatment (Null) grew with the introduction of new media as shown in Figure 8 below. It exhibited similar growth to a control culture that was grown using mid-log *E. coli* (Control).

Two Week Reinfection

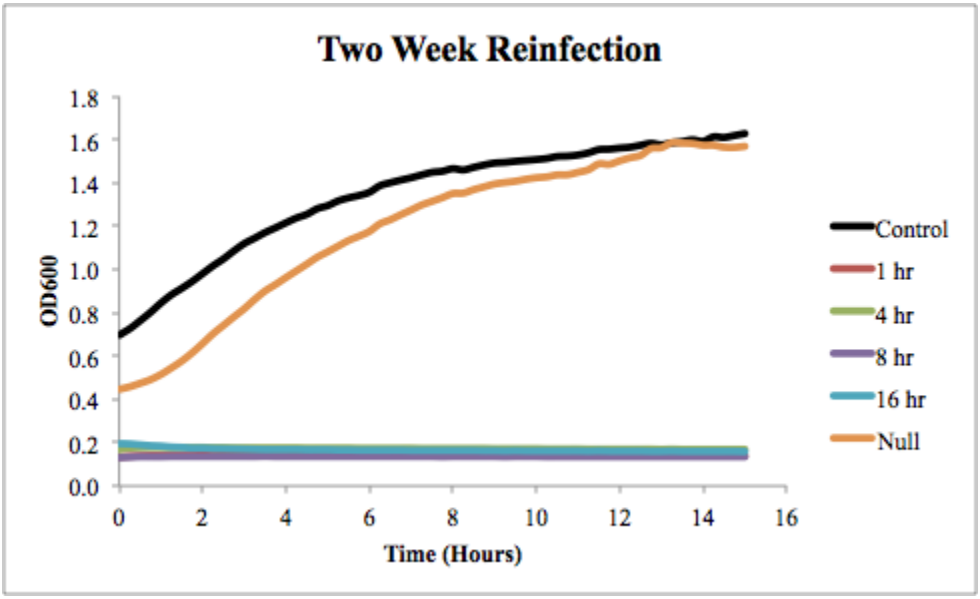

Figure 9. The control (new *E. coli* grown in new media), null (old *E. coli* grown in new media), 1-hour, 4-hour, 8-hour and 16-hour wells were reinfected with *E. coli* two weeks after the time dosage experiment. The growth course for the newly introduced *E. coli* is plotted above.

Well	**Well**	**P-value**
Control	Null	0.000*
Control	1 hr	0.000*
Control	4 hr	0.000*
Control	8 hr	0.000*
Control	16 hr	0.000*

Table 9. Statistical analysis for two week reinfection analysis. P-values indicate statistical significance of OD difference between wells. Asterisks indicate significant trials.

In addition, no growth was observed for all wells previously treated with 0.05M Ga-DFO and re-inoculated with mid-log *E. coli*. There is a significant difference in OD [F(5, 359)= 844.423, p=0.000] between treatment conditions. Post hoc tests show a significant difference between the control well and the null, 1 hour, 4 hour, 8 hour and 16 hour wells (p=0.000).

Mammalian Cell Culture Treatment

	CHO Control Flask		CHO Test Flask	
	Cell Count [Viable cells/ml (*10^6)]	Viability (%)	Cell Count [Viable cells/ml (*10^6)]	Viability (%)
Initial (before dilution/inoculation)	0.92	96.6	0.79	97.5
1 Hour	0.88	96.8	3.22	31.3
24 Hours	1.84	98.2	1.88	22.4

Table 10. Viable cell counts and percentage of total cells which are viable for CHO cells treated with 0.005M Ga-DFO and controls.

	HEK Control Flask		HEK Test Flask	
	Cell Count [Viable cells/ml (*10^6)]	Viability (%)	Cell Count [Viable cells/ml (*10^6)]	Viability (%)
Initial (before dilution/inoculation)	0.12	85.1	0.12	85.1
24 Hours	0.35	96.6	0.007	58.3

Table 11. Viable cell counts and percentage of total cells which are viable for HEK cells treated with 0.005M Ga-DFO and controls.

24 hours after introducing treatment, cell counts and percentage of cells which are viable decreased significantly for both CHO-S and HEK cell cultures. As seen in Tables 10 and 11,

CHO-S cell viability dropped from 97.5% to 31.3% within 1 hour and was 22.4% by 24 hours. In contrast, the CHO-S control flask had viabilities of 96.6%, 96.8%, and 98.2% at the same times. HEK cell viability and cell counts were 0.12 Viable cells/ml (*10^6) with 85.1% viability for both control and treated cultures before treatment or dilution. After 24 hours, HEK control increased to 0.35 Viable cells/ml (*10^6) with 96.6% viability while treated HEK decreased to 0.007 Viable cells/ml (*10^6) with 58.3% viability.

Images of CHO-S cell culture were taken for both control and treated samples at 1 and 24 hours after passage and treatment occurred (Figure 10: A-D). Figures 10: A-B were taken of the control flask at 1 and 24 hours respectively and appear as expected for a CHO-S culture. Figures 10: C-D were taken of CHO-S cultures treated with Ga-DFO at 1 hour and 24 hours after passage/treatment. It is clear that the treated cultures appear quite different under microscope than the control cultures.

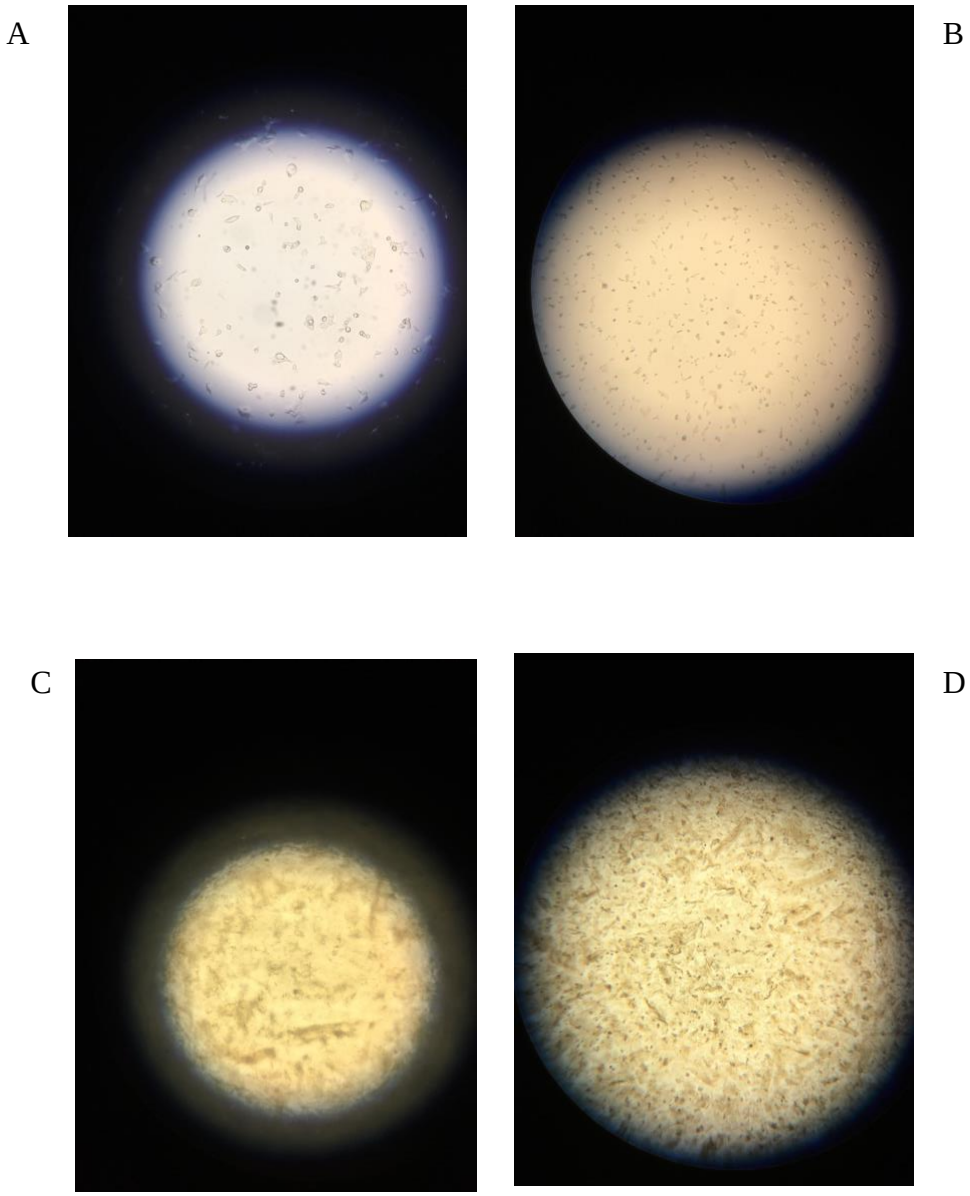

Figure 10: A-D. Enhanced images of CHO-S suspension. A: Image of control CHO-S suspension at 1 hour after passaging. B: Image of control CHO-S suspension at 24 hours after passaging. C: Image of CHO-S suspension + 0.005M Ga-DFO 1 hour after passaging/treatment. D: Image of CHO-S suspension + 0.005M Ga-DFO 24 hours after passaging/treatment.

Discussion

In Phase I of our experiment, we found the MIC estimate of Ga-DFO for *E. coli, E. aerogenes, S. aureus,* and *S. typhimurium* to be 0.005M Ga-DFO, 0.05M Ga-DFO, 0.005M Ga-DFO, and 0.05M Ga-DFO, respectively. For each of our strains, post hoc tests showed a

significant difference between 0.005M Ga-DFO and 0.005M Ga(III) (p=0.000), and 0.05M Ga-DFO and 0.005M Ga(III) (p=0.000) (Figure and Tables 1-4). This indicates DFO complexation mediates the antibacterial activity of Ga(III), thus validating the siderophore-uptake model for the development of new antibiotics. These findings dispute the supposed toxic activity of Ga(III) ions alone, but support previous findings regarding the antibacterial of Ga-DFO.

In an attempt to refine the MIC estimate of *E. coli*, we determined the MIC estimate of Ga-DFO to be 0.0031M (Figure 5). Previous research has found the MIC of Ga-DFO to be much lower for other bacteria (Minandri et al., 2014), but it is possible that different media compositions may have altered the activity of Ga-DFO. A previously unaddressed question was the efficacy of Ga-DFO in non-iron depleted media, since iron could theoretically disrupt the bonds between Ga(III) and DFO. We found that administration in complex media did require a higher concentration Ga-DFO in order to inhibit growth. However, this decrease in activity may or may not be attributed to iron levels in the growth media.

Time-Dependent Dosage

The time course experiment showed that Ga-DFO is effective at inhibiting growth of *E. coli* when added at any point during the bacterial growth curve. There was a significant difference in OD between *E. coli* alone and *E. coli* with Ga-DFO added at 1 hour [$F(1,367)=2398.092$, $p=0.000$], 4 hours [$F(1,343)=1900.089$, $p=0.000$], 8 hours [$F(1,311)=528.862$, $p=0.000$] and 16 hours [$F(1,247)=6672.418$, $p=0.000$]. Therefore, the treatment is feasible for application in a real world setting where the growth stage of bacteria is unknown. The bacteria in the 24 hour treatment was contaminated so this experiment should be

replicated to ensure that all time points are not contaminated. Future studies should replicate this experiment with several bacterial strains in order to confirm that the effect is conserved.

Two Week Media Transfer

After 2 weeks of incubation, 1, 4, 8 and 16 hour treatment wells were plated using fresh media and no observable colonies were formed. In contrast, the null group (2-week old *E. coli* with no drug treatment) grew with the introduction of new media. This suggests that the Ga-DFO treatment has bactericidal activity and killed *E. coli* instead of just inhibiting further proliferation. If the treatment had bacteriostatic activity, bacteria would be able to grow once it was introduced to new media and nutrients.

Two Week Reinfection

When *E. coli* was reintroduced to a treated culture 2 weeks after treatment, growth was significantly inhibited at 1, 4, 8 and 16 hours. Specifically, OD remained approximately 0, indicating that no growth occurred. This suggests a lack of metabolic processes that are able to inhibit the effects of Ga-DFO. These results show that Ga-DFO offers great promise as a spray antibacterial treatment, since it remains effective over the course of two weeks and would therefore not require re-application. Future studies should investigate how long exactly Ga-DFO remains effective, since longevity of treatment has many implications for the feasibility of a commercial product and what it could be used for. They should also make sure that the treatment remains stable after reinfection with various strains of bacteria, and even several bacteria at the same time in order to simulate a real life antibacterial application.

Since the treatment remained effective after the initial reinfection, we can infer that the treated cells lysed and released Ga-DFO into the media to be uptaken by any new bacteria that are introduced. However, this theory is not proven by our present study. Another potential avenue for future investigations could find the mechanism by which Ga-DFO remains effective over time. Specifically, we could measure how many cells lyse after Ga-DFO treatment using methods like live/dead cell staining. In order to understand the dynamics of Ga-DFO uptake, we could determine the amount of Ga-DFO that is present in the media twelve hours after treatment and after two weeks. It would also be useful to determine whether there is a dose dependent effect of Ga-DFO's effectiveness after reinfection.

Mammalian Cell Culture Treatment

The drops seen in both Cell Count and Cell Viability suggest that Ga-DFO may be toxic to both CHO-S and HEK cells. While post treatment reads were only done at 1 and 24 hours for CHO cells and 24 hours for HEK cells, it is possible that cell death continues to occur after 24 hours unless cells are removed from treated culture. One area of confusion in the cell count data is the massive spike in cell count number for treated CHO-S cells at 1 hour. It is likely that the ViCell cell counter may have been unable to differentiate the CHO-S cells from small molecules in the treatment. However, this does not invalidate the finding because from 1 hour to 24 hours, cell count and viability both still decrease from the peaked number considerably.

The results of Phase III of the experiment fail to confirm the findings of prior literature which suggest that Ga-DFO is safe in the presence of mammalian cells. The rapid decrease in both cell viability percentage and number suggest Ga-DFO may be toxic to both HEK and CHO-S cells. However it can not be confirmed that the Ga-DFO is causing the direct death of the mammalian cells rather than a byproduct of its addition. For this reason the results of Phase III

are inconclusive. It is possible that the mammalian cells died due to a change of culture pH when Ga-DFO was added.

Future Directions

Mammalian Cell Culture

While the addition of a 0.005M concentration of Ga-DFO in phase III killed both HEK and CHO-S cell cultures, it is unknown whether the treatment itself killed the cells or if cell death was a product of the acidic environment caused by adding the treatment. During the mammalian cell culture treatment when Ga-DFO in DMEM was added to an HEK culture also in DMEM, the media turned from red to yellow as seen in Figure 11 below. DMEM contains phenol red as a pH indicator, showing that adding the treatment increased the acidity of the media. A future experiment could add a pH neutralizer known to be non-toxic to mammalian cells.

Figure 11. Color of DMEM media with HEK cells with Ga-DFO added (left) and without (right). Color indicative of phenol red interacting with pH of media.

An additional future experiment to better test the effect of Ga-DFO on mammalian cells could be to rerun the experiment from Phase III but to passage cells out of treated CHO-S and HEK cultures after a given amount of time to fresh media without treatment and observe if the cells resume growth or not. This experiment could give greater insight into how Ga-DFO affects HEK and CHO-S cells.

Antibiotic Resistance

A gallium-siderophore complex has the added benefit of potentially avoiding resistance because it manipulates pre-established iron-transport pathways. Also, it is predicted that resistance will not be able to combat a gallium-siderophore complex as quickly as it had with antibiotics, if at all, because Ga(III) is a multi-target drug that is predicted to impair several Fe(III)-dependent functions. Resistance mechanisms such as target mutation, drug modification or development of alternative metabolic pathways are unlikely to develop in bacteria to overcome Ga(III) inhibition (Minandri et al., 2014). While no claims can definitively be made about the potential for resistance to a gallium-siderophore complex, the manipulation of iron-transport pathways and impairment of several vital functions makes treatment likely to remain effective longer than traditional antibiotics.

These predictions were supported by studies investigating the evolutionary potential for resistance against a gallium treatment. Researchers compared gallium to two conventional antibiotics and used *Galleria mellonella* used to assay virulence in acute infections in caterpillar hosts (Ross-Gillespie et al., 2014). The results showed that cultures treated with the antibiotics rose significantly in resistance whereas gallium retained effectiveness over time (Ross-Gillespie et al., 2014). This indicates that gallium is less susceptible to the development of resistance than conventional antibiotics.

Previous studies indicate that bacteria may have less evolutionary potential to develop resistance to gallium treatment compared to traditional antibiotics (Ross-Gillespie et al., 2014; Minandri et al., 2014). The combination of nutrient starvation and introduction of harmful metal ions by gallium-conjugated siderophore complexes represents a promising strategy for reducing rates of bacterial resistance. Mutations that prevents uptake of an iron-substitute would likely be lethal, since the mutation would also prevent the uptake of iron (Minandri et al., 2014). Since

potential mechanisms of avoiding the treatment would be maladaptive, it is possible that the usual development process of antibiotic resistance would be cut off before it begins. Instead of a few bacterial cells acquiring traits that help them survive and then reproducing to pass on those traits, bacteria may be unable to mutate in a way that allows them to survive. Metal-siderophore complexes could represent an alternative to traditional antibiotics that can be used to address the growing problem of antibiotic resistance.

However, further studies are necessary to confirm these findings. Since Ga-DFO shows promise as antibacterial treatment, future investigations should investigate the ability of bacteria to evolve resistance to both free gallium and Ga-DFO. This would require testing reinfecting free gallium treatment and the Ga-DFO treatment against a strain of bacteria over a considerably longer period of time to give the bacteria enough time to develop, if at all possible, resistance to the treatment.

Antimicrobial Treatment

Since results are inconclusive about the efficacy of Ga-DFO in mammalian cell culture, future experiments should investigate using the treatment as an antimicrobial spray as discussed above. These experiments could include design and optimization of application technique, testing the effectiveness of the spray against different bacterial species or on different surfaces, and investigating how long the spray can effectively inhibit bacterial growth. Formulation of a Ga-DFO topical treatment could also be an important application of the technology. Similar experiments should be conducted to test the efficacy of a topical ointment, particularly investigating the stability of the complex itself over time and in various temperatures. If this

ointment proves to be stable and effective in many conditions, it could be an attractive treatment for use by the military or veterinary care.

Potency Treatment

Results indicate that the Ga-DFO treatment is potent against *E. coli*. Future experiments should investigate the degree of potency. The administration of the free gallium treatment and the Ga-DFO treatment should be done in different environmental conditions. These environmental conditions could include under a UV light, on a solid surface, and in disks. Since Ga-DFO shows promise as a treatment against biofilm bacteria in initial studies (Banin et al., 2008), future experiments should also investigate its effectiveness against biofilms containing a wide spectrum of bacteria in order to mimic a real-life application.

Appendices

A1: CAS protocol

This protocol is adapted from Schwyn and Neilands (1987) and scaled up and modified for convenience. Addition of iron is omitted because this CAS solution is intended to detect gallium. Since gallium concentrations will be variable, no gallium is added to the stock solution.

Materials:

1 L volumetric flask

Chrome Azurol S 50% (Aldrich 199532)

Hexadecyltrimethylammonium bromide (HDTMA) (Sigma H6269)

Piperazine (make unknown)

Hydrochloric Acid, concentrated (make unknown)

CAS Reagent Preparation:

1. Rinse volumetric flask with 6 M HCl
2. Rinse volumetric flask with DI water 3 times
3. Dissolve 43.07 g piperazine in DI water in volumetric flask
4. Add 62.5 mL concentrated HCl to flask
5. Add 0.18 g CAS to flask
6. Add 0.218 g HDTMA to flask
7. Parafilm top of flask and carefully invert until all phases mix and bubbling subsides. Bleed air from top of flask if parafilm is bulging outward.
8. Filter resulting solution (0.22 micron is sufficient, non-sterile paper filters work as well)
9. Store filtered solution in opaque plastic bottle in a cool area

A2.1: Minimum Inhibitory Concentration Range

Materials:

Gallium trichloride, solid

DFO mesylate salt, solid

Autoclaved DI water

LB

2.2X LB

24-well plate

Early log-phase culture of *E. coli*

Early log-phase culture of *S. Typhimurium*

Early log-phase culture of *S. Aureus*

Early log-phase culture of *E. Aerogenes*

Night Before

1. Inoculate 50 mL flask bacteria 18 hours before planned start of experiment.
2. Add thawed *E.coli* stock to 50 mL LB. Shake at 37℃ and 250 RPM.
3. Repeat step 2 for *S. Typhimurium, S. Aureus* and *E. Aerogenes*

Day of

4. Transfer 10 mL of *E. coli* culture from the 50 mL LB flask to the 500 mL LB flask 6 hours before planned start of the experiment
5. Measure the OD of the log phase culture and dilute to an OD of 0.3 with PBS
6. Repeat steps 4-5 for *S. Typhimurium, S. Aureus* and *E. Aerogenes*
7. Add 4.5 mL of 1M Ga-DFO solution to 5.5 mL of 2.2XLB and then perform a 1:10 dilution with LB to produce 0.05M Ga-DFO treatment
8. Perform an additional 1:10 dilution with LB to produce 0.005M Ga-DFO treatment

9. Add 4.5 mL of 1M $Ga(Cl)_3$ solution to 5.5 mL of 2.2XLB and perform two 1:10 dilutions with LB to produce 0.005M $Ga(Cl)_3$ solution

Plate Preparation

10. Plate on a 24-well plate accordingly,

	1	2	3	4	5	6
A	1.13mL 2.2XLB + 1.25 mL PBS + 0.125 mL *E. coli*	-	2.38mL 0.05M Ga-DFO + 0.125 mL *E. coli*	2.38mL 0.005M Ga-DFO + 0.125 mL *E. coli*	2.38mL 0.005M $Ga(Cl)_3$ + 0.125 mL *E. coli*	1.13mL 2.2XLB + 1.25 mL PBS
B	1.13mL 2.2XLB + 1.25 mL PBS + 0.125 mL *S. Typhimurium*	-	2.38mL 0.05M Ga-DFO + 0.125 mL *S. Typhimurium*	2.38mL 0.005M Ga-DFO + 0.125 mL *S. Typhimurium*	2.38mL 0.005M $Ga(Cl)_3$ + 0.125 mL *S. Typhimurium*	1.13mL 2.2XLB + 1.25 mL PBS
C	1.13mL 2.2XLB + 1.25 mL PBS + 0.125 mL *S. Aureus*	-	2.38mL 0.05M Ga-DFO + 0.125 mL *S. Aureus*	2.38mL 0.005M Ga-DFO + 0.125 mL *S. Aureus*	2.38mL 0.005M $Ga(Cl)_3$ + 0.125 mL *S. Aureus*	1.13mL 2.2XLB + 1.25 mL PBS
D	1.13mL 2.2XLB + 1.25 mL PBS + 0.125 mL *E. Aerogenes*	-	2.38mL 0.05M Ga-DFO + 0.125 mL *E. Aerogenes*	2.38mL 0.005M Ga-DFO + 0.125 mL *E. Aerogenes*	2.38mL 0.005M $Ga(Cl)_3$ + 0.125 mL *E. Aerogenes*	1.13mL 2.2XLB + 1.25 mL PBS

Microplate Setup

1. Activate the microplate reader
2. Select the 24-well plate
3. Select 37℃, slow shake, run for 15 hours and collect points every 15 minutes

4. Click “Read”

A2.2: Minimum Inhibitory Concentration

Materials:

Gallium trichloride, solid

DFO mesylate salt, solid

Autoclaved DI water

LB

1.33X LB

24-well plate

Early log-phase culture of *E. coli*

Night Before

1. Inoculate 50 mL flask bacteria 18 hours before planned start of experiment.
2. Add thawed *E.coli* stock to 50 mL LB. Shake at 37℃ and 250 RPM.

Day Of

3. Transfer 10 mL of culture to 500 mL flask 6 hours before planned start of experiment.
4. Weigh out 1.148 g of DFO mesylate and 0.36g Ga(Cl3) and add to 2.50mL of autoclaved DI water and 7.50mL 1.33X LB
5. Measure the OD of the log phase culture and dilute to an OD of 0.3 with PBS

Plate Preparation

6. Plate on a 24-well plate accordingly,

	1	2	3	4	5	6
A	2.50mL LB	2.40mL LB	2.5mL of Ga-DFO	1.20mL LB	1.20ml LB	1.20mL LB
B	-	-	1.20mL LB LB	1.20mL LB LB	1.20mL LB LB	1.20mL LB LB

C	2.50mL LB	2.40mL LB	1.20mL LB	1.20mL LB	1.20mL LB	1.20mL LB
D	-	-	1.20mL LB	1.20mL LB	1.20mL LB	1.20mL LB

7. On the same plate, perform 1:2 dilutions as follows,

	1	2	3	4	5	6
A	-	-	2.5mL of Ga-DFO	1.25mL from well D3	1.25mL from well D4	1.25mL from well D5
B	-	-	1.25mL from well A3	1.25mL from well A4	1.25mL from well A5	1.25mL from well A6
C	-	-	1.25mL from well B3	1.25mL from well B4	1.25mL from well B5	1.25mL from well B6
D	-	-	1.25mL from well C3	1.25mL from well C4	1.25mL from well C5	1.25mL from well C6

8. On the same plate, add the following,

	1	2	3	4	5	6
A	-	0.10mL *E. coli*	1.20 LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*
B	-	-	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*
C	-	0.10mL *E. coli*	1.20 mL LB + 0.05mL	1.20 mL LB + 0.05mL	1.20 mL LB + 0.05mL *E.*	1.20 mL LB + 0.05mL *E.*

			E. coli	*E. coli*	*coli*	*coli*
D	-	-	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*	1.20 mL LB + 0.05mL *E. coli*

Microplate Setup

1. Activate the microplate reader
2. Select the 24-well plate
3. Select 37℃, slow shake, run for 15 hours and collect points every 15 minutes
4. Click “Read”

A3: CFU Protocol

Materials:

Gallium trichloride, solid

DFO mesylate salt, solid

Autoclaved DI water

LB

1.33X LB

PBS

96 well plate

Early-log phase *E. coli*

Night Before

1. Inoculate 50 mL flask bacteria 18 hours before planned start of experiment. Add thawed *E.coli* stock to 50 mLLB. Shake at 37℃ and 250 RPM.

Day Of

1. Weigh out 0.657g of DFO mesylate and 0.176g Ga(Cl3) and add to 2.50mL of autoclaved DI water and 7.50mL 1.33X LB.
2. Transfer 10 mL of culture to 500 mL flask 6 hours before planned start of experiment.
3. Harvest 10 mL of cell culture. This is time 0.
4. 5 minutes after time 0:

   Take a 10 uL sample from the mixed culture and dilute 1:10 in 90 mL PBS. Do 9 further serial dilutions to end with a final dilution of 10^{-10}. Plate 10 uL from each dilution in an LB plate.

   *This can be conducted in a 96 well plate.

5. Repeat for time points 15 and 30 minutes after time 0.
6. Incubate all plates at 37℃ for 12 hours. After incubation, remove and count single colonies.

A4: Time-Dependant Dosage

Materials:

Gallium trichloride, solid

DFO mesylate salt, solid

Autoclaved DI water

LB

1.33X LB

24-well plate

Early log-phase culture of *E. coli*

Night Before

1. Inoculate 50 mL flask bacteria 18 hours before planned start of experiment.

   Add thawed *E.coli* stock to 50 mLLB. Shake at 37℃ and 250 RPM.

Day of

2. Transfer 10 mL of culture to 500 mL flask 6 hours before planned start of experiment.
3. Weigh out 0.574g of DFO mesylate and 0.18g Ga(Cl3) and add to 2.50mL of autoclaved DI water and 7.50mL 1.33X LB
4. Measure the OD of the log phase culture and dilute to an OD of 0.3

Plate Preparation

5. Plate on a 24-well plate accordingly,

	1	2	3	4	5	6
A	2.50mL LB	2.40mL LB + 0.1 mL *E. coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*

B	2.50mL LB	2.40mL LB + 0.1mL *E. coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*
C	2.50mL LB	2.40mL LB + 0.1mL *E. coli*	1.25mL LB	1.25mL LB	1.25mL LB	1.20mL LB + 0.05mL *E.coli*
D	2.50mL LB	2.40mL LB +0.1mL *E. coli*	1.25mL LB	1.20mL LB + 0.05mL *E.coli*	1.20mL LB + 0.05mL *E.coli*	-

6. Run the plate in the microplate reader for 1 hour

7. After 1 hour, add the Ga-DFO treatment accordingly,

	1	2	3	4	5	6
A	-	-	1.25mL Ga-DFO Treatment	-	-	-
B	-	-	1.25mL Ga-DFO Treatment	-	-	-
C	-	-	1.25mL Ga-DFO Treatment	-	-	-
D	-	-	-	-	-	-

8. After 4 hours (from start of experiment), add the Ga-DFO treatment accordingly,

	1	2	3	4	5	6

A	-	-	-	1.25mL Ga-DFO Treatment	-	-
B	-	-	-	1.25mL Ga-DFO Treatment	-	-
C	-	-	-	1.25mL Ga-DFO Treatment	-	-
D	-	-	-	-	-	-

9. After 8 hours (from start of experiment), add the Ga-DFO treatment accordingly,

	1	2	3	4	5	6
A	-	-	-	-	1.25mL Ga-DFO Treatment	-
B	-	-	-	-	1.25mL Ga-DFO Treatment	-
C	-	-	-	-	1.25mL Ga-DFO Treatment	-
D	-	-	-	-	-	-

10. After 16 hours (from start of experiment), add the Ga-DFO treatment accordingly,

	1	2	3	4	5	6

A	-	-	-	-	1.25mL Ga-DFO Treatment	-
B	-	-	-	-	1.25mL Ga-DFO Treatment	-
C	-	-	-	-	1.25mL Ga-DFO Treatment	-
D	-	-	-	-	-	-

9. After 24 hours (from start of experiment), add Ga-DFO treatment accordingly,

	1	2	3	4	5	6
A	-	-	-	-	-	-
B	-	-	-	-	-	-
C	-	-	-	-	-	-
D	-	-	1.25mL Ga-DFO Treatment	1.25mL Ga-DFO Treatment	1.25mL Ga-DFO Treatment	-

Microplate Setup

1. Activate the microplate reader
2. Select the 24-well plate
3. Select 37℃, slow shake, run for 24 hours and collect points every 15 minutes
4. Click “Read”

*note: at the end of the experiment, place the microplate into an incubator at 37℃

A5: Two Week Media Transfer/Reinfection

Materials

24-well plate from time-dosage experiment

LB

Early log-phase culture of *E. coli*

Sterile loops

LB plates

CFU Analysis

1. After 2 weeks, remove the plate used in "Time-Dependent Dosage Experiment" from the incubator
2. Using a sterile loop, spread the contents in the treatment wells on seperate LB plates
3. Place the plates in an incubator at 37℃

Plate Preparation

1. Using a 24-well plate, plate accordingly,

	1	2	3	4	5	6
A	2.50mL LB	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture
B	2.50mL LB	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture	1.15mL LB + 0.1 mL *E. coli* + 1.25 mL old culture
C	2.40mL LB + 0.1mL *E.*	1.25 mL LB + 1.25 mL	1.15mL LB + 1.25 mL	1.15mL LB + 1.25 mL	1.15mL LB + 1.25 mL	1.15mL LB + 1.25 mL

	coli	old culture	old culture	old culture	old culture	old culture
D	2.40mL LB + 0.1mL *E. coli*	1.15mL LB + 1.25 mL old culture	1.15mL LB + 1.25 mL old culture	1.15mL LB + 1.25 mL old culture	1.15mL LB + 1.25 mL old culture	

Microplate Setup

1. Activate the microplate reader
2. Select the 24-well plate
3. Select 37℃, slow shake, run for 24 hours and collect points every 15 minutes
4. Click “Read”

A6: Mammalian Cell Culture Experiment

Materials:

4x disposable T25 filtered flasks

3x disposable 125ml filtered shake flasks

2x 10ml disposable conical tubes

101ml Freestyle Serum Free CHO media + 0.008M L-Glut

Suspension-adapted Freestyle Chinese Hamster Ovary (CHO-S) cells

59ml DMEM with 10% FBS and 1% Penicillin-Streptomycin + 0.008M L-Glut

Fibercell Human Embryonic Kidney (HEK) Adherent cells

6x ViCell disposable test tubes

10mL Trypsin

Ultrapure type 1 (milli-Q) water

Gallium trichloride, solid

DFO mesylate salt, solid

2x 50ml disposable conicals

2x 50ml sterile filter conicals

Making Ga-DFO for CHO-S cells:

1. In a fume hood, add 0.1985g Gallium Chloride(III) to 2.5mL ultrapure type 1 water in a 50ml disposable conical
2. Add 0.6289g Desferrioxamine to the same conical, close the conical and mix till all precipitate is dissolved

3. In a Biosafety cabinet attach a 50mL sterile filter conical to the 50mL conical of Ga-DFO and attach the air pump to the filter. With the air pump on, flip the conical and hold until all liquid has switched conicals. Remove the air pump and empty conical
4. Add 7.5mL Freestyle Serum Free CHO media + 0.008M L-Glut to the 50ml conical

Making Ga-DFO for CHO-S cells:

1. In a fume hood, add 0.1440g Gallium Chloride(III) to 7.5mL ultrapure type 1 water in a 50ml disposable conical
2. Add 0.4562g Desferrioxamine to the same conical, close the conical and mix till all precipitate is dissolved
3. In a Biosafety cabinet attach a 50mL sterile filter conical to the 50ml conical of Ga-DFO and attach the air pump to the filter. With the air pump on, flip the conical and hold until all liquid has switched conicals. Remove the air pump and empty conical
4. Add 22.5mL DMEM with 10% FBS and 1% Penicillin-Streptomycin + 0.008M L-Glut to the 50ml conical

CHO-S Cell Line Inoculation:

1. Retrieve Freestyle Serum Free CHO media + 0.008M L-Glut and place it in a 37℃ water bath
2. Place a pipette gun, multiple pipette gun tips, one disposable 125ml filtered shake flask, and a waste container inside a sterile biosafety cabinet
3. Retrieve cell banked CHO-S Freestyle cells from dewar and thaw cells in 37℃ water bath and hand
4. Retrieve warmed CHO media and place both the thawed cells and media into the sterile biosafety cabinet

5. Add 30mL CHO media to the filtered shake flask using the pipette gun
6. Add the thawed CHO cells to the same flask using the pipette gun
7. Place the shake flask in the CO2 incubator at 37℃ on a shaker at 100 RPM for 1-3 days until cells are at appropriate seeding density

HEK Cell Line Inoculation:

1. Retrieve DMEM with 10% FBS and 1% Penicillin-Streptomycin and place in a 37℃ water bath
2. Place a pipette gun, multiple pipette gun tips, a disposable T25 filtered flask, a disposable 10mL conical tube, and a waste container inside a sterile biosafety cabinet
3. Retrieve cell banked HEK Fibercell adherent cells from dewar and thaw cells in 37℃ water bath and in hand
4. Retrieve warmed DMEM and place both the thawed cells and media into the sterile biosafety cabinet
5. Add 5mL DMEM to the 10mL conical
6. Add HEK cells to the conical using a pipette gun
7. Centrifuge the conical at 200 RCF for 5 minutes
8. In the biosafety cabinet, remove the supernatant from the centrifuged conical and resuspend the pellet in 5mL fresh DMEM
9. Using pipette gun, transfer the resuspended pellet in media to the T25 flask
10. Place the T25 flask in the incubator at 37℃ for 1-3 days until cells are at ~95% confluency

CHO-S Cell Line Passage:

1. Retrieve CHO media and place in a 37℃ water bath

2. Retrieve the shake flask with CHO cells from 1-3 days prior and place in a sterile biosafety cabinet
3. Place 2 disposable 125mL filtered shake flasks, 1 ViCell disposable test tube, a pipette gun, a waste container, and multiple pipette gun tips into the biosafety cabinet
4. Remove .5ml of media and cells from the inoculated shake flask and transfer it into the ViCell test tube
5. Use the ViCell cell counter to take a preliminary cell count of the media in the test tube
6. Using the preliminary cell count, determine how much the inoculated CHO culture would need to be diluted to achieve a seeding density of 3*10^5 in 30ml of media
    a. Sample calculation: 3mL of 3*10^6 cell count solution (preliminary reading) + 27mL of solution = 30mL of 3*10^5 cell count solution
7. Retrive the CHO media from the water bath and place it in the biosafety cabinet
8. Use the pipette gun to transfer the calculated amount of fresh media into each of the two empty shake flasks
9. Use the pipette gun to transfer the calculated amount of inoculated media (with cells) into each of the shake flasks
10. Place both newly inoculated shake flasks in the CO2 incubator at 37℃ on a shaker at 100 RPM for 24 hours and dispose of the old shake flask

<u>HEK Cell Line Passage:</u>

1. Retrieve the T25 flask with HEK Fibercell cells from 1-3 days prior
2. Examine the T25 flask under a microscope to check for ~95% confluency. If confluency is less than 95%, allow cells to continue growth in the incubator before passaging
3. Retrieve the DMEM and place it in a 37℃ water bath

4. Place a pipette gun, multiple pipette gun tips, 9 disposable T25 filtered flasks, a disposable 10mL conical tube, 2.5ml Trypsin, and a waste container inside a sterile Biosafety Cabinet
5. Remove and dispose of the media in the inoculated T25 flask
6. Add 2.5ml of trypsin to the same T25 flask
7. Place the T25 flask with Trypsin in the CO2 incubator at 37℃ for 3 minutes
8. After 3 minutes add 1.25ml of DMEM to the incubated T25 flask in the biosafety cabinet and pipette the supernatant up and down the side of the flask which cells have adhered to
9. Remove the supernatant and transfer it to an empty 10mL conical
10. Centrifuge the conical at 200 RCF for five minutes
11. After 5 minutes, in the biosafety hood, remove and dispose of the supernatant from the centrifuged conical
12. Resuspend the pellet at the bottom of the conical in 10ml fresh DMEM
13. Add 4mL of fresh DMEM into each of the 3 empty T25 flasks
14. Transfer 1mL of media from the conical to each of the same 3 T25 flasks
15. Place the T25 flasks in the CO2 incubator at 37℃ for 24 hours

<u>CHO-S Cell and HEK Cell Treatment:</u>

1. 24 hours after passaging, retrieve the 3 T25 flasks and 2 shake flasks from the CO2 incubator at 37℃
2. Place the DMEM and CHO media in a 37℃ water bath
3. Place the 3 T25 flasks, 2 shake flasks, 3 ViCell test tubes, a pipette gun, a waste container, ultra-pure type 1 water, both stocks of Ga-DFO, and multiple pipette gun tips into a sterile biosafety cabinet

4. Label 1 T25 flask ‘non-diluted/treated’, 1 T25 flask ‘24 hour treated’, and 1 T25 flask ‘24 hour diluted’
5. Remove and dispose of the media in the T25 flask labeled ‘non-diluted/treated’
6. Add to the same T25 flask 2.5ml of trypsin
7. Place the T25 flask with Trypsin in the CO2 incubator at 37℃ for 3 minutes
8. After 3 minutes add 1.25ml of DMEM to the incubated T25 flask in the biosafety cabinet and pipette the supernatant up and down the side of the flask which cells have adhered to
9. From the same T25 flask remove 0.5ml of media and transfer it into a ViCell test tube to serve as an initial cell count for all HEK flasks before treatment
10. Transfer .5ml of media from each of the inoculated shake flasks into ViCell test tubes for pre-treatment cell counts
11. Use the ViCell cell counter to take cell counts of all 3 test tubes
12. In the biosafety cabinet, inoculate the shake flask labeled ‘treated’ with 3mL of .055M GaDFO
13. Add 3mL of .0133M GaDFO to the T25 flask labeled with ‘treated’
14. Inoculate the remaining shake flask with 3ml CHO media
15. Dispose of the T25 flask labeled ‘non-diluted/treated’
16. Place the remaining 2 T25 flasks and 2 shake flasks in the CO2 incubator at 37℃ with the 2 shake flasks on a shaker at 100 RPM

Data Collection:

1. At 24 hours after step 14 in CHO-S Cell and HEK Cell Treatment was completed above, complete the following steps
2. Place the DMEM in a 37℃ water bath

3. Place both shake flasks, 4 ViCell test tubes, a pipette gun, a waste container, the 2 T25 flasks, and multiple pipette gun tips into a sterile biosafety cabinet
4. Remove and dispose of the media in both T25 flasks
5. Add to the T25 flasks 2.5ml of trypsin each
6. Place the T25 flasks with Trypsin in the CO2 incubator at 37℃ for 3 minutes
7. After 3 minutes add 1.25ml of DMEM to the incubated T25 flasks in the biosafety cabinet and pipette the supernatant up and down the side of the flask which cells have adhered to
8. From the same T25 flasks remove .5ml of media and transfer it in to 2 ViCell test tubes
9. Transfer .5ml of media from each of the shake flasks into 2 ViCell test tubes
10. Use the ViCell cell counter to take cell counts of all 4 test tubes
11. Dispose of all flasks

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