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Drug Interactions with Glutaredoxin Orthologues

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Honors Research Project

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Introduction

Glutaredoxin (GRX), a small enzymatic protein in the thioredoxin family, consists of about 100 amino acid residues. It can serve a number of functions within different cells, such as DNA synthesis (as cells depend on ribonucleotide reduction to form deoxyribonucleotides), regulation and distribution of iron, and protein repair mechanisms. GRX is involved in redox-regulation, as it catalyzes the reduction of protein disulfides [1]. GRXs act as reducing agents by transferring electrons to other proteins and forming a disulfide bridge by way of the thiol (SH) groups of their Cysteine residues. This enzyme contains an active site disulfide bond that is oxidized by substrates (usually other proteins) and reduced non-enzymatically by its cofactor glutathione [2]. Glutaredoxins can defend against antibiotic activity by reducing proteins such as dehydroascorbate, peroxiredoxins, and methionine sulfoxide reductase. For example, GRX plays a significant role in antibiotic resistance in bacterial *Pseudomonas aeruginosa* cells.

Pseudomonas aeruginosa (*P. aeruginosa)* is a pathogen that causes infections. It is active in patients with pneumonia and cystic fibrosis. *P. aeruginosa* is especially harmful/deadly in cystic fibrosis—caused by lung infections—patients, because *P. aeruginosa* infections are not easily treated. Unfortunately, 18% of the infections that are treated come back as a result of the pathogen's inherent antibiotic resistance [3]. Thus, it is necessary to gear treatment of these infections toward the development of drugs (or medicinal fragments) that are capable of killing the infection, as well as prevent the chance of its return/relapse.

GRXs act to repair oxidatively damaged amino acid residues of proteins with disordered disulfides. Thus, inhibition of GRX can result in a number of physiological problems, including impairment of innate immune function. Neutrophil activity is deregulated by reactive oxygen species (ROS) that elicit irreversible glutathionylation of cytoplasmic actin; inhibiting movement

and bactericidal/antimicrobial function of these essential phagocytic components of innate immunity. As a result, neutrophils depend on the catalytic activity of GRX1, which is responsible for deglutathionylation of its actin filaments [4]. While release of ROS is a natural process associated with phagocytosis, abnormally high concentrations of ROS can lead to life threatening illnesses (such as septic shock). Consequently, a fair amount of pharmacological studies have focused on mechanisms that inhibit the negative regulation of cellular function by ROS [5, 12]. Thus, GRX is a potential target of antioxidant, as well as antimicrobial pharmacology.

In this study, *P. aeruginosa* GRX (PaGRX) as well as the GRX orthologue of *Brucella melitensis* (BrmGRX) were chosen targets of fragment based drug discovery (FBDD) using Nuclear Magnetic Resonance (NMR) techniques. This strategy involves screening a large library

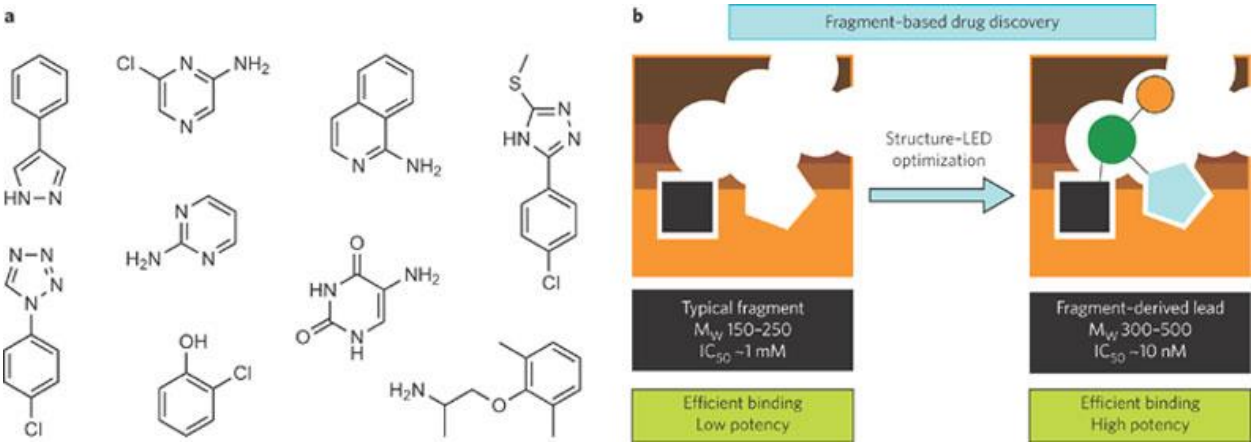


Figure 1. In FBDD, High Throughput Screening (HTS) is often utilized to assess a large library of potential drug fragments that bind to a target protein. Fragment hits are usually between 150 and 250 kDa; examples of typical fragments are presented in **a.** Once small fragment binders are identified, they can be chemically altered to increase their interaction with the target protein; and ultimately raise their binding affinity for the target protein (**b.**). Taken from reference 13.

of small fragment molecules to determine which interact (or bind) with the three orthologous proteins of interest, namely human GRX1 (hGRX), PaGRX, and BrmGRX. While orthologous proteins are similar in sequence and shape, subtle differences in their structure can be exploited to select for fragments that bind tightly to bacterial orthologues, and have little to no interaction

with the human species. Once these lead fragments are identified, they can be analyzed (to determine their binding affinity) and modified to increase their binding affinity. They can be assayed against bacterial cells (*P. aeruginosa* and *B. melitensis*) and eukaryotic cells (human cells form cancerous tissue) to determine a therapeutic range for future use in clinical trials to help treat diseases (or infections) that depend on the catalytic action of bacterial GRXs.

To assess binding affinity of target drug fragments for the GRX proteins, it is necessary to isolate PaGRX, BrmGRX and hGRX1 from bacterial cells (BrmGRX (C70S) and hGRX1(C8S) used in this study are cysteine-to-serine mutant forms of the proteins to enhance their stability and solubility for NMR screening). This is accomplished by extracting these proteins from transformed bacterial cells. The protein is then purified using fast protein liquid chromatography (FPLC), which takes advantage of the target protein's strong affinity, relative to other unwanted compounds or proteins, for a specific resin that it binds to, to isolate the target protein from other cellular contaminants.

Once a small fragmental drug molecule, capable of binding both BrmGRX and PaGLRX, is developed, it is necessary to test the ability of the drug to inhibit function of the bacterial protein without harming the human orthologue. This can be accomplished by performing minimum inhibitory concentration (MIC) and MTT (3-*(4,5-Dimethylthiazol-2-Yl)*-2,5-Diphenyltetrazolium Bromide) assays on the drug fragments to determine the amount of drug needed to kill the bacterial cells without killing human host cells.

A MTT assay is a calorimetric assay that can be used to measure eukaryotic cell viability. More specifically, it measures the enzymatic activity (of NAD(P)H-dependent cellular oxidoreductase) within a cell responsible for reducing the dye, MTT, to insoluble formazan [6].

A MIC assay, measures the lowest concentration of an antimicrobial (such as ampicillin) capable of inhibiting the growth of a microorganism after overnight incubation. An MIC assay assesses a microorganism's ability to resist antimicrobial activity [7].

These assays are important in this research because they can test the efficacy of lead fragments, (such as RK395 and RK207, which have high binding affinity for PaGRX and BrmGRX respectively) identified as a result of FBDD and NMR screening, to bind to the target proteins of a specific infectious species, with very minimal, if any at all, side-effects inflicted on human cells.

Materials and Methods

Protein Purification

In order to isolate each of the three proteins (hGRX1, PaGRX, BrmGRX) needed for NMR screening, the proteins had to be expressed by transformed bacterial cells, and purified using liquid chromatography. Majority of the reagents and equipment utilized for protein purification were purchased from Fischer Scientific (Pittsburgh, PA). General safety tips are included in the appendix.

General Bacterial Transformation

Competent bacterial cells (roughly 50 µL of cells weakened through exposure to high slat concentrations, such as Ca^{+2}) were extracted from a liquid nitrogen dewer, and thawed on ice. One microliter of the appropriate DNA plasmid solution was added to the cell solution, and mixed. The cell/DNA mixture was placed back on ice for 15 minutes, heat shocked for 30 sec at

42°C, and incubated back on ice for an additional 2 minutes. One half of a milliliter of warmed SOC (super optimal broth with catabolite repression) media was added to the tube, which was then shaken (at 260 rpm; shaking prevents cell sedimentation and distributes media evenly throughout solution) for 30 minutes at 37°C. Two different concentrations of cell mixture were plated onto two separate LB-Agar Petri dishes with the appropriate antibiotic (either ampicillin, kanamycin, or carbenicillin). One-hundred microliters of the inoculum was transferred to one plate, and 400 µL to the other (cells were transferred using proper aseptic technique; including ethanol flame sterilization).The plates were placed in a 37°C incubator overnight.

Protein Extraction

A small amount transformed bacterial cells were added to a culture tube (using proper aseptic technique) containing 5 mL of warmed, autoclaved, Lauria-Bertani (LB; from MB Biomedicals; Santa Ana, CA; see appendix for recipe)) media and 5 microliters of antibiotic (Kanamycin/ampicillin for hGRX and PaGRX and Carbomycin for BrmGRX). The culture tube was incubated overnight at 37°C (at 260 rpm). One liter of M9 minimal growth medium was prepared using the protocol included in the appendix. The M9 solution was filtered using 1 L sterile cup filters, and divided equally between two 2800 mL conical flasks. Twenty-five milliliters of M9 solution were extracted from each flask and transferred to a 250 mL conical flask. Two milliliters of the cultured bacterial cells were added to the 250 mL conical flask, and the flask was shaken and incubated for 12-15 hours at 37°C and 260 rpm. A small amount of M9 solution was pipetted into a cuvette for use as a reference for absorbance measurements.

Fifteen milliliters of the inoculum in the 250 mL conical flask was transferred into each of the 2800 mL flasks. The flasks were incubated for 4-5 hours at 37°C (260 rpm), or until the absorbance of the inoculum was around 0.5 (O.D. readings past 0.5, indicate cells transitioning from growth phase to lag phase, or induction of cellular apoptosis) at 600 nm wavelength (absorbance readings were recorded at one hour intervals). Once an absorbance of 0.5 was obtained (a 30 µL sample was taken for the SDS-PAGE gel), the flask were inoculated at 18°C, and 375 µL of IPTG (a lactose metabolite that induces protein expression) were added to the mixture (a gel sample of this mixture was taken). The flasks were then shaken overnight at 18°C. The solution in each flask was divided equally into 250 mL centrifuge tubes and spun for 5 minutes using the 16.25 rotor at 8000 rpm and 4°C, using the Department of Chemistry's Beckman-Coulter (Brea, CA) high speed centrifuge. The supernatant was discarded, and the cell pellets remained in the tubes and were kept on ice (or stored at -80°C).

Buffer solutions (A and B) were prepared (for lysis and liquid chromatography) using the protocol included in the appendix, and filtered using millipore filter paper. The cells were suspended in a total of 30 mL of buffer A by vortexing each tube. The suspension was transferred to a 50 mL falcon tube. Roughly 30 µL each of three protease inhibitors (benzamidine, leupeptin, and PMSF) were added to the cell suspension. The solution was incubated on ice for roughly 20 minutes to allow the added components to react. The cells were lysed using the department's French press (Thermo Scientific, Waltham, MA; with caution, at least three rounds were completed each time to get the lysate as clear as possible; and a sample of the lysate was taken for the gel). The French press exerts over 1,000 psi units of force on the cell mixture to rupture the cell walls and release the internal content of the cells, which includes target GRX proteins. The lysed cells were centrifuged using the 25.5 rotor, at 13000 rpm, at 4°C

for 15 minutes. The proteinaceous supernatant was transferred to a new falcon (30 µL of supernatant and pellet were taken for gel) tube by filtering it through a 25mm GD/X syringe filter.

Protein Purification

The resultant protein sample was further purified using an AKTA Fast protein liquid chromatography (FPLC; GE Healthcare) machine. Protein samples were injected into a HisTrap IMAC (Immobilized Metal ion Affinity Chromatography) 5 mL nickel column (GE Healthcare) to optimize purification of the histidine-tagged proteins, and separate uncleaved (tagged) protein from the crude lysate containing all soluble cleaved protein. Uncleaved protein was eluted in the flow through; cleaved protein was eluted by Buffer B from the IMAC nickel column. The cleaved protein was collected in 5mL intervals into fraction tubes. The fraction tubes containing the highest absorption peak, at 280 nm, were collected in a falcon tube (30 µL samples of the flow through and peak fractions were taken for the gel).

Two liters of buffer A were prepared at pH 8, without imidazole, and used to dialyze the protein overnight (to remove the imidazole from the protein solution). Thermo Fisher Scientific SnakeSkin® pleated dialysis tubing with a molecular weight cut off of 3 kDa was used. For hGRX1 only, 500 µL of TEV (tobacco Etch virus) protease were added to the solution (HR V 3C protease was used for BrmGRX; and a size exclusion column was used for PaGRX), to remove His tag fusion partners, this reaction was incubated overnight in the fridge. A second column was run on the dialyzed protein mixture (gel samples for flow through and peak samples were taken as well),

without his tag containing fusion partners. Thus, the target protein was collected in the FT. Fractions were collected, for proteins that still contained histidine tag fusion partners, in a falcon tube and dialyzed in fresh dialysis buffer (the same used previously), to remove the imidazole in the protein sample.

An Amicon stir cell (Millipore) was used to concentrate the protein. Absorbance readings, at 280 nm, were taken and concentration of the protein was determined using the Beer-Lambert Law. Once the desired concentration was obtained (for NMR it should be between 0.3 and 0.5 mM), the protein was stored in the nitrogen dewer or NMR tubes.

SDS PAGE

Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) is a technique used to separate protein mixtures based on their size. SDS, an anionic detergent, was used to even the charges between the proteins and denature them. This detergent binds tightly to proteins, and once the proteins were coated with SDS they all become relatively uniform in charge and thus could be reasonably distinguished based on their size. The gel in SDS-PAGE is more resistant to the movement of larger molecules due to frictional forces [8]. Using a voltmeter, an electric current was sent through the gel containing the protein; all of the molecules traveled in the same direction because they are negatively charged, and thus travel toward the anode.

Each protein sample was loaded into a gel made of polyacrylamide, and an electric field was applied to the gel to separate proteins based on length, or molecular weight. Proteins moved

down the gel toward the anode at a rate inversely proportional to their size (thus larger proteins moved slower than smaller ones).

It was necessary to use gel electrophoresis to confirm the presence of the target proteins (hGRX, BrmGRX and PaGRX), and confirm their purity based on the number of bands displayed on the gel. All pellet samples taken for the gel were resuspended in equal amounts of sterile water and 2X SDS- 10% β-mercaptoethanol (BME) loading buffer (which contained Coomassie brilliant blue dye, to track protein bands during gel-electrophoresis). An equal amount of 2X SDS-10% was added to the liquid samples (thus if 30 µL of solution was in the Eppendorf tube, then 30 µL of 2XSDS-10% BME were added). The samples were mixed, capped and placed on the heating block for 5 minutes at 95°C. The samples were then agitated using the "washboarding" technique, and spun in the microcentrifuge. The gel box was prepared by making 1 L of 1X running buffer and putting the gel in place. Ten microliters of each sample was loaded into each well, along with 5 uL of prestained protein ladder. The gel was run at 200 V for roughly 30-45 min (varied depending on the size of the target protein). The gel was removed and placed in a gel box. It was rinsed with water and covered with blue-silver dye, and placed on the rocker platform overnight. The gel was destained the following day, whereupon protein bands could visibly be distinguished and compared.

Eukaryotic Cell Culturing

Bringing up frozen cells

The cell line (4th generation ATCC H-1975) used for eukaryotic cell culturing and MTT assays were adherent cells derived from cancerous lung tissue. Prior to use, the cells were kept frozen (in liquid nitrogen at -80 degrees C) in 5% DMSO (dimethyl sulfoxide), RPMI-1640 medium (from ATCC; with 10% fetal bovine serum), and trypsin. RPMI-1640 media was warmed to roughly 37°C, and placed in the laminar flow hood (every item was wiped down with 70% EtOH before being placed in the hood).

Ten milliliters of media were pipetted into each of the cell culturing plates (1 plate/ cryotube) used. Two cryotubes containing the 4th generation H-1975 cell line were extracted from the liquid nitrogen dewer and thawed in a 37° water bath (special care was taken to avoid submerging the tubes past the cap). The cryotubes were rinsed with EtOH and immediately brought to the hood. The solution in each tube was then transferred to each of the two plates. The plates were swirled, covered, and checked under an inverted microscope to confirm the presence of the cells, which were incubated at 37° C. The following day, the media was replaced with 10 mL of fresh RPMI-1640. The cells were allowed to grow until they were needed.

Splitting Cells

When cells were 80-90% confluent (adhered to 80-90% of the cell plate), they were split into 2-4 new culture plates containing 10 mL of warmed media. The media the cells were in, prior to splitting, was removed/sucked up using a vacuum pump and Pasteur pipette. Two milliliters of trypsin protease were added to the cells on each plate (and swirled), and allowed to incubate for roughly 2 minutes (or until cells had begun to lift from the plate) in the 37° C incubator. The plate was then returned to the hood, and 2 mL of media were added to each plate.

The cells were further removed from the bottom of the plate by pipetting the solution on the plates up and down while holding the plate on a slight angle. The cell suspension from each plate was collected in a 10 mL pipette; roughly 0.5-1 mL of the cell solution was transferred to each new plate containing 10 mL of fresh media. The plates were properly labeled (cell line type, media used, date, and your initials), checked under the microscope, and transferred to the incubator. The remaining cell solution was either bleached and discarded, or used for an MTT assay.

Freezing Cell Lines

It was necessary to preserve a portion of the propagating cells for future use, and to have as backup in the event that the cells being cultured were to get contaminated (e.g. with bacteria or fungus). The cells were first treated with trypsin, and collected in a 15 mL falcon tube. They were then centrifuged at 150g for 8 minutes. The supernatant was carefully removed, and the pellet of cells were suspended in 1 mL media/cryotube (4 in this case). Five percent DMSO (0.24 mL of autoclaved DMSO) was added to the solution, and 1 mL of the solution was pipetted into each cryotube, which were immediately placed in the liquid nitrogen dewer.

Mic Assay

The procedure for assessing the MIC of an antibiotic drug starts with the preparation of an antibiotic solution, agar dilution plates, and an inoculum. The next steps are inoculation, incubation, and interpretation of results [7].

During the Minimum Inhibitory Concentration (MIC) assays bacterial cells were grown in Mueller-Hinton (MH) broth and exposed to varying concentrations of antimicrobial agents. The optical density (O.D.), or absorbance, of each sample was measured to compare cell viability to a 0.5 Mcfarland standard suspension. MIC assays were conducted against a number of antibiotics (including Ruthenium(III) chloride ($RuCl_3$), dichloro(p-cymene)ruthenium(II)dimer (or Ru-cymene), RK395, and more) following the protocol outlined by Barry, A. et. al. [9]

Assays were performed using either the microdilution and/or agar dilution plate method. Several culture tubes (for microdilution), or plates, were prepared with MH broth/or agar containing varying concentrations of the antimicrobial agent. The tubes and plates were inoculated with a standard suspension of bacterial cells (0.5 Mcfarland standard) so that each test would start with the same initial concentration of microorganisms. The cells were incubated at 37°C for up to 24 hours, and cell viability (or turbidity) was quantified to determine the MIC of various antibiotics.

E.coli cells were initially inoculated in 5 mL of LB medium (incubated at 37°C and 260 rpm). The medium was later switched to autoclaved MH broth, and the E.coli cells were cultured on MH agar plates (from a 10 fold diluted 0.5 Mcfarland standard suspension, with an O.D. of 0.096 at 625 nm). Similarly, *P. aeruginosa* (PA01) was inoculated in 5 mL of LB medium (at 37°C and 260 rpm). After 18 hours of incubation, the medium for *P. aeruginosa* (PA01) was switched to MH broth, and cultured on MH agar plates (from a 10 fold diluted 0.5 Mcfarland standard suspension, with an O.D. of 0.087 at 625 nm).

E. coli cells were taken from the culture plate and added to 5 mL of saline (sterilized, 0.85% NaCl; incorporated to emulate physiological salt concentrations). This solution had a

Mcfarland 0.5 standard O.D. of 0.104 at 625 nm. The solution was diluted 10^{-7}, 10^{-6}, 10^{-3} and 10^{-2} fold. One-hundred microliters of each diluted sample was plated separately onto 4 different plates.

Sterile tubes were placed in a rack and labeled for each lead compound/antibiotic (one tube was used as a growth control and only contained bacteria and broth, another was used as a sterility control and only contained broth; all antibiotics were diluted in MH broth). Assays were performed in replicates for each concentration of antibiotic.

A total of 5 antimicrobial agents were assayed, (including RuB207, amide, Ru-amine, $RuCl_3$ and Ru-cymene), for the first assay. One milliliter of sterile broth (MH broth) was added to each tube. One milliliter of antibiotic/ lead compound (concentration = 128 ug/mL) was added to the first tube, making its concentration 64 ug/mL. One milliliter was extracted from the first tube and transferred to the second tube (32 ug/mL); serial dilution was continued until the 7th tube was reached (the 8th and 9th tube were controls and contained no antibiotic). *E. coli* cells were extracted from their culture plate and added to 5 mL of saline equivalent to a McFarland 0.5 standard (with an O.D. of 0.101 at 625 nm). One-hundred microliters of the *E.coli* cell suspension were transferred into a tube containing 9.9 mL of MH broth. Two-hundred microliters of this solution were added to each of the seven tubes containing fragment compounds and MH broth (for all 5 lead compounds; making each cell suspension 10^4 - 10^5 CFU/mL). The rack was placed in the incubator for roughly 24 hours.

Similarly, 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6} fold dilutions were prepared for *P. aeruginosa* (PA01). The 10^{-4}, 10^{-5} and 10^{-6} fold dilutions were grown on plates. Cell viability was assessed 18-24 hours later for both dilutions.

Higher concentrations of lead fragments ($RuCl_3$ H2O, Ru-cymene, ampicillin(Amp), and carbenicillin(Carb); amp and carb are known inhibitors of *P. aeruginosa* and *E.coli* cell proliferation) were used for a 2nd assay with both bacterial species; the concentrations used were 2.56, 1.28, 0.64, 0.32, 0.16, 0.08 mg/mL). A standardized 0.5 McFarland inoculum was prepared using the direct colony suspension method. The 0.5 McFarland suspension was diluted (with an O.D. of 0.104 for *E. coli* and 0.103 for *P. aeruginosa* at 625 nm) in a 1:150 solution of MH (i.e. 66 µL of the standard solution was mixed with 9.934 µL of MH both). As a result, each tube contained approximately 1 X 10^6 CFU/mL.

Fifteen minutes after the inoculum was standardized, 500 uL of the adjusted inoculum were added to each tube containing 1 mL of lead compound in the dilution series (and a positive control tube containing only broth), and mixed. This resulted in a final inoculum of 5 X 10^5 CFU/mL. Based on macrodilution results, agar dilutions were plated for select dilutions of each lead compound, and cell viability was assessed the following day.

A third assay was performed using 1 mg/mL, 0.9 mg/mL, 0.8 mg/mL, 0.7mg/mL, 0.6 mg/mL and 0.5 mg/mL concentration of Ru-cymene against *E. coli* and *P. aeruginosa* (PA01) (with the same carbenicillin concentrations for *P. aeruginosa*).

The last MIC test was performed using 10^4-10^5 CFU/ml of *P.aeruginosa* against varying concentrations of RK395 (1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL)

MTT Assay

MTT assays are designed to measure cell viability and proliferation, in this case, in the presence of a potential drug/lead compound (RK 395, RK395- acrylic acid coupled (RK395-

ACP), and RK207). During this assay, yellow tetrazolium salt or MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide) reagent is reduced by live (or metabolically active) cells, through dehydrogenase enzymes; by products of this reaction include NADH and NADPH. After the reaction takes place, formazan, a purple product is solubilized and can be quantitated using spectroscopy. (Travigen's TACS MTT Cell proliferation Assay). One-hundred microliters of cultured H-1975 cells (diluted in RPMI-1640 media) were aliquoted into each of the wells of a 96 well plate. Fifty microliters of diluted lead compound were added to each column of wells (except the first column which contained 4 blank wells and 4 growth control wells with just media and cells), and incubated overnight. Ten microliters of MTT reagent were then added to each well, and the plate was incubated for roughly 4 hours (to allow the reduction of MTT to formazan). Detergent was added to each well to solubilize the formazan dye for spectroscopic measurements. Absorbance values were taken using a microplate reader at 540 nm. Cultured cells were treated with trypsin, and spun down using a centrifuge at 150 rpm, and 20°C for 20 minutes. Prior to spinning down the cells, a 100 µL sample was taken for the hemocytometer apparatus, which was used to determine the amount of live cells in the solution. The 100 µL of cells were mixed with 100 µL of trypan blue stain. Ten microliters of that solution were added to the hemocytometer, and cells in each of the 4 large quadrants were counted and averaged. Using a hemocytometer calculator, the number of live cultured cells could be determined. Roughly 5,000 cells were added to each well. Four hours after adding the MTT reagent, cells were checked under the inverted microscope to confirm a change in color, signifying the formation of the formazan dye. After the dye was solubilized, and absorbance reading were taken, the absorbance values for each concentration of lead fragment (MTT assays were conducted on RK395, RK395 ACP, and RK207) was averaged (a total of 8 assays were done at each

concentration) and the absorbance for the blank was subtracted from the averaged values. Absorbance versus concentration of lead fragment were plotted (absorbance values between 0.75 and 1.25 were significant for untreated cells).

STD NMR

Each of the target proteins were analyzed using NMR (all ligand-based NMR spectra were conducted on a Varian 750 MHz instrument with a cryoprobe) and screened against a library of 463 small fragment compounds to search for selective hits (that would ideally bind to the bacterial orthologues, with minimal binding to hGRX). Using Saturation Transfer Difference NMR, chemical shifts could be assessed (when proteins were mixed with fragments versus when they were dissolved in solution alone) to determine which fragments bind with greatest affinity, or have significantly small dissociation (K_d) constants. These hits, or lead fragments, could then be modified to increase their binding affinity by adding additional chemical components to their structure.

Results

Results obtained for this research were collaboratively collected by multiple group members working under Dr. Leeper's instruction/guidance; including Ram Khattri, Caroline Davis, and Dan Morris.

Protein Purification

Once each of the three orthologous proteins was expressed, it was ultimately purified using AKTA FPLC. Figures 2 and 3 display examples of FPLC results obtained during purification of BrmGRX. The uncleaved protein from the first column run were pooled form fractions 5-8, which are associated with the peak absorbance of eluted protein (represented by the blue plot in the figure). The protein was purified a second time, as shown in figure 2. The change in purity is represented by less eluted protein (smaller absorbance peak at 280 nm) in comparison to the same peak in figure 1. There is also less cleaved flow through (the first peak observed for the absorption curve at 280 nm), confirming increased purity of the sample. The protein was concentrated using Amicon stri cell, and its final concentration was determined by measuring its absorbance at 280 nm (A_{280} = 0. 369), and using the Beer-Lambert equation (A = Clε, were ε =

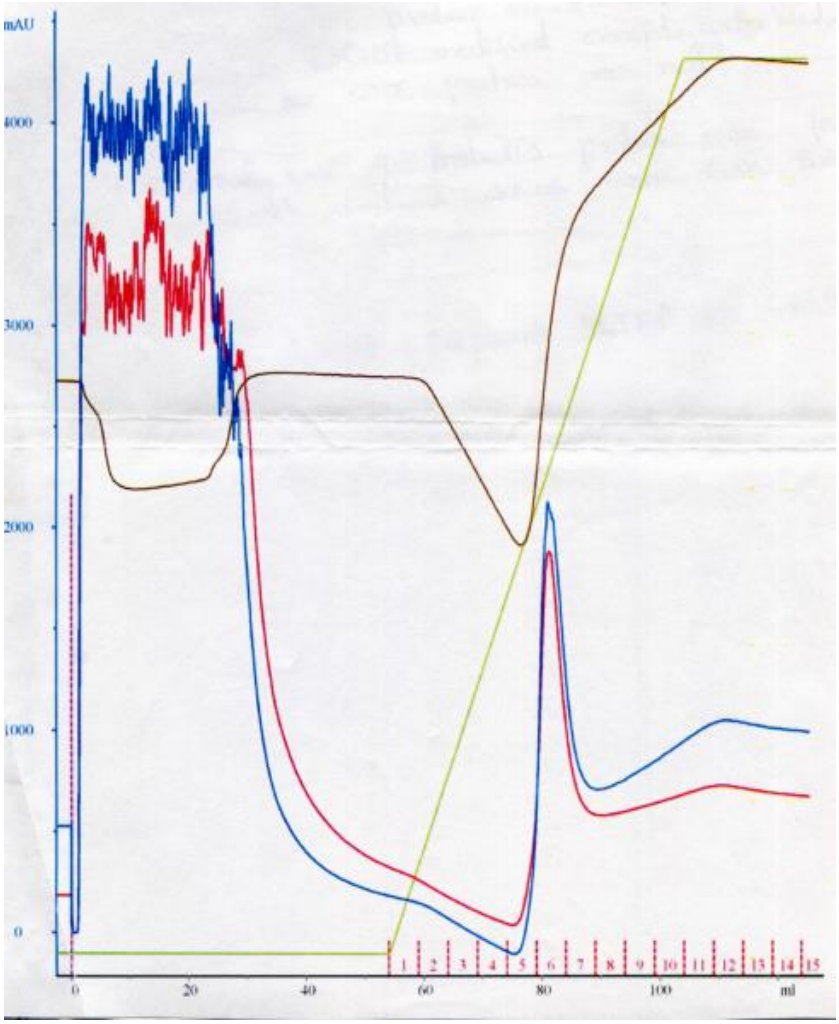


Figure 2. Sample FPLC data for target protein (BrmGRX). Once the protein was extracted from bacterial cells, it was injected into the HisTrap IMAC nickel column and separated from untargeted protein and other cellular contaminants. The first peak (at 280 nm) represents the flow through of crude lysate. The 2nd peak represents the his-tagged BrmGRX protein that was bound to the column; the fractions from this peak were

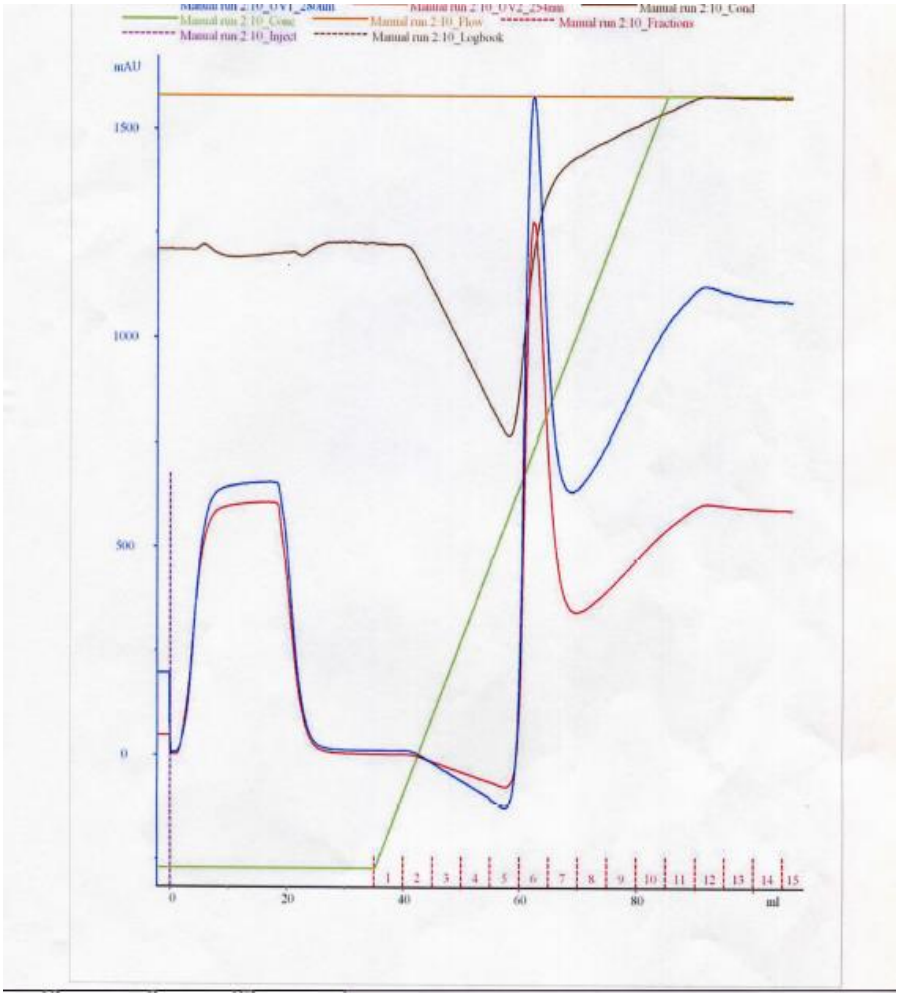


Figure 3. The dialyzed BrmGRX was injected into the nickel column a second time. This time the first peak at 280 nm represents the flow through of cleaved BrmGRX, which was collected in a falcon tube. The 2nd peak represents the residual his-tagged BrmGRX protein that was not cleaved by a protease.

4470 M^{-1} cm^{-1}). The final concentration was determined to be 9.25 mg/ml; SDS-PAGE was employed to further confirm the purity of the sample.

SDS-PAGE

The following figure is an example of a stained gel used to confirm the synthesis of target GRX orthologue proteins. BrmGRX is a 9.6 kDa protein with 88 amino acid residues, lane 3 confirmed the presence of this purified protein. HGRX1 is an 11.70 kDa protein with 106 amino acid residues, its successful synthesis is confirmed in lanes 8 and 10. PaGRX is a 9.2 KDa protein with 84 amino acid residues, its presence and purity is confirmed in lane 2. [10,11] Lane 1 contains the standard ladder of known protein sizes and was compared with the bands produced from various protein samples extracted during protein purification. As expected, multiple bands appear from samples contaminated with other proteins (lanes 4-7) such as

samples taken after centrifuging lysed recombinant bacterial cells. Purity of protein samples can be confirmed by comparing the number, and size, of bands from samples extracted earlier in the protein purification procedure versus the final purified product. Lane 4, which was extracted from a protein sample produced after centrifuging lysed bacterial cells, shows more bands that vary in size confirming that the sample contains more than the desired PaGRX product; whereas the band for purified PaGRX in lane 2 displays one band (although faint bands above the easily distinguished band representing PaGRX suggest that the final product may not be completely pure) that was separated in the area of the gel that one would expect PaGRX to end up.

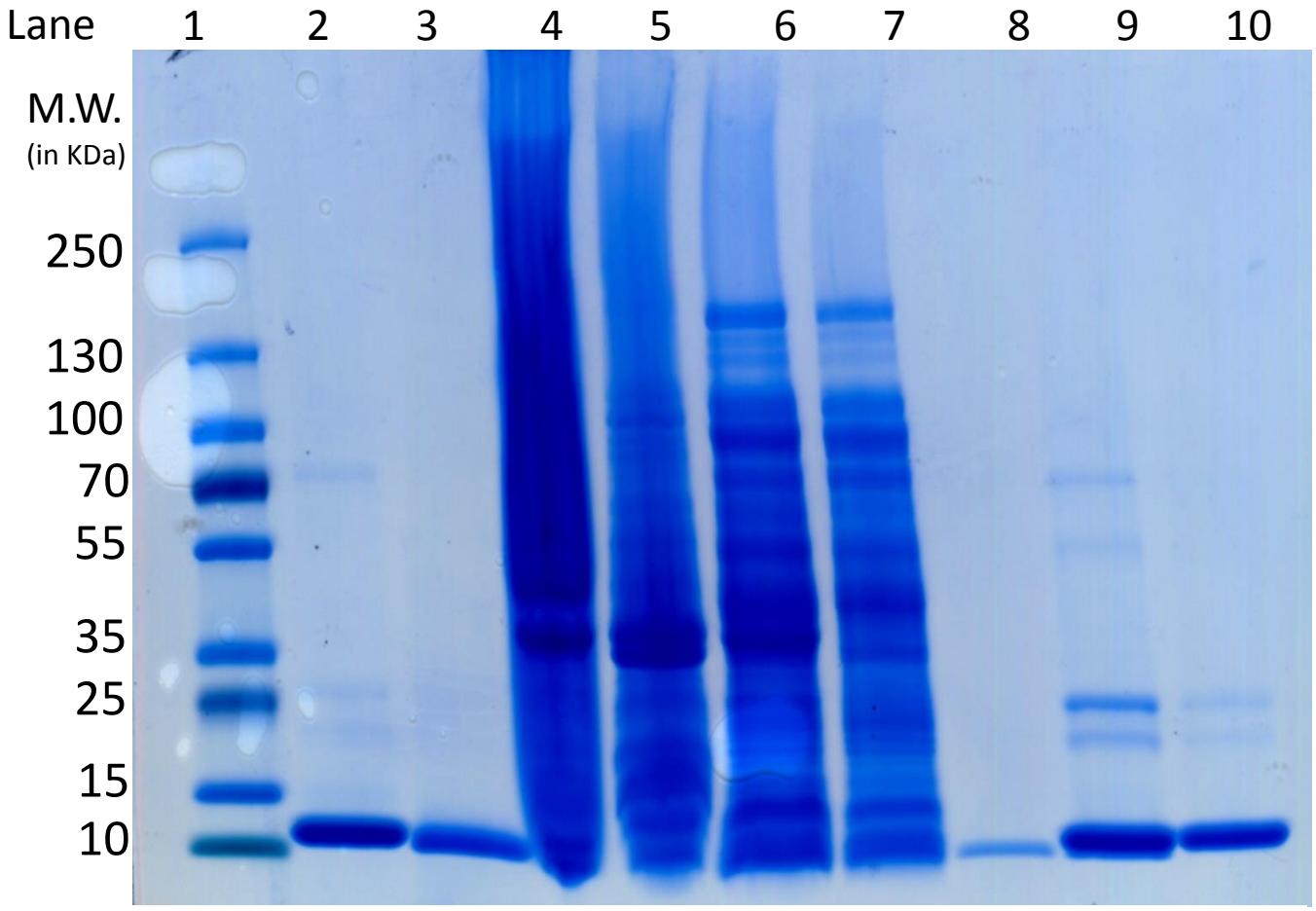


Figure 4. Picture of SDS-PAGE with blue silver dye staining. Lane 1: Ladder (PageRuler Plus Prestained Protein Ladder); Lane 2: PaGLRX; Lane 3: 15N Braba C70S (cleaved); Lane 4: 15N PaGLRX after centrifugation; Lane 5: 15N hGRX after centrifugation; Lane 6: 15N PaGLRX 1st column F.T. ; Lane 7: 15N hGRX F.T. after 1st column; Lane 8: 15N hGRX cleaved after 2nd column; Lane 9: 15N hGRX uncleaved; Lane 10: 15N hGRX uncleaved after 2nd column.

MIC Assay

MIC assays were conducted (using *E. coli* and *P. aeruginosa* (PA01) cells) against a number of candidate fragments and/or antibiotics including $RuCl_3$, Ru-cymene, Ampicillin, Carbenicillin, Rub207, amide, Bu-amine and RK395. The first assay conducted against Rub207, amide, Bu-amine, RuCl3 and Ru-cymene was unsuccessful. Both cell lines grew significantly in the presence of the lead fragments ranging in concentration from 1 ug/mL to 64 ug/mL. Thus, the subsequent assay was conducted against $RuCl_3 \cdot H2O$, Ru-cymene, Ampicillin, and carbenicillin at higher concentrations. The following table presents the average O.D. values obtained for *E. coli* and *P. aeruginosa* (PA01) cells exposed to varying concentrations of antibiotic.

	Concentration (mg/mL)	2.56	1.28	0.64	0.32	0.16	0.08	The control
E. coli	$RuCl_3$	**2.435**	0.596	1.961	1.944	1.858	N/D	1.659 (No AB)
	Ru-cymene	0.001	0.032	1.272	1.565	1.759	N/D	
	Ampicillin	0.000	0.000	0.000	0.000	0.000	N/D	
P. aeruginosa	$RuCl_3$	**2.423**	0.519	1.857	1.951	1.860	1.852	1.664 (No AB)
	Ru-cymene	0.000	0.004	1.658	1.773	1.743	1.793	
	Carbenicillin	0.000	0.000	0.000	0.000	0.641	1.499	

Table 1. Absorbance (O.D.) values of bacterial cells inoculated with varying concentrations of $RuCl_3$, Ru-cymene, amp, and carb at 625 nm; after 19 hours of incubation at 37°C and 260 rpm. Because $RuCl_3$ has an intense color at higher concentrations, O.D. values in bold are erroneous.

E. coli cells inoculated with 2.56 and 1.28 mg/mL of both $RuCl_3$ and Ru-cymene, and 1.28 mg/mL of Ampicillin were plated on agar plates by transferring 100 uL of each mixture to five separate plates. A similar process was done with *P. aeruginosa* (including the 0.64 mg/mL inoculum, and using carbenicillin instead of ampicillin). The following results were obtained:

Concentration of antibiotic (mg/mL)	2.56 of $RuCl_3$	1.28 of $RuCl_3$	2.56 of Ru-cymene	1.28 of Ru-cymene	1.28 of Ampicillin
E. coli	No Growth	No Growth	No Growth	too many colonies to count	No Growth

Table 2**.** Only plates with 100 µL of Ru-cymene at 1.28 mg/mL contained colonies.

Concentration of lead fragment/ antibiotic (mg/mL)	2.56 of $RuCl_3$	1.28 of $RuCl_3$	0.64 of $RuCl_3$	2.56 of Ru-cymene	1.28 of Ru-cymene	0.64 of Ru-cymene	1.28 mg/mL of carbenicillin
P. aeruginosa	No growth	No Growth	too many colonies to count	390 colonies	about 800 colonies	too many colonies to count	21 colonies

Table 3. Plates with 100 µL of 0.64 mg/mL of $RuCl_3$ and 100 µL of, 2.56, 1.28, and 0.64 mg/mL of Ru-cymene contained bacterial colonies. The results from this table and Table 2 were used for the subsequent assay.

Based on the results obtained from the previous assay, an additional assay was performed against Ru-cymene at the following concentrations; 1, 0.9, 0.8, 0.7, 0.6, and 0.5 mg/mL with both *E. coli* and *P. aeruginosa* (PA01) cells. After 18 hours of incubation at 37°C (and 260 rpm), it was discovered that 0.7 mg/mL of Ru-cymene was the MIC for *E. coli* and 1 mg/mL was the MIC for *P. aeruginosa*, as the following table suggests:

Concentration (mg/mL) of compound	1 mg/mL	0.9 mg/mL	0.8 mg/mL	0.7 mg/mL	0.6 mg/mL	0.5 mg/mL	Positive control
Ru-cymene for *E. coli*	0.001	0.001	0.002	0.002	1.269	1.426	1.792 (w/o Ab)
Ru-cymene for *P. aeruginosa*	0.002	0.060	1.033	1.435	1.470	1.491	1.801 (w/o Ab)
Carbenicillin for *P. aeruginosa*	0.000	-0.004	-0.003	-0.004	-0.003	-0.003	-0.003

Table 4. O.D. values obtained from assay against *E. coli* and *P. aeruginosa* with Ru-cymene (and carb against *P. aeruginosa)*

The most recent MIC test was performed using 10^4-10^5 CFU/ml of *P.aeruginosa* against different concentrations of RK395. Absorbance (O.D.) measurements were taken at 625 nm for each sample, after 22 hours of incubation at 37°C and 260 rpm. The following results were obtained:

Test tube #	1	2	3	4	5	6	7	8	9	10	11	12	13
[RK395] µg/ml	1024	512	256	128	64	32	16	8	4	2	1	GC	SC
Abs.	0.329	1.636	1.819	1.830	1.823	1.778	1.822	1.735	1.701	1.775	1.827	1.935	0

Table 5. MIC assay against *P.aeruginosa* with varying concentrations of RK395(GC=growth control; SC=sterility control)

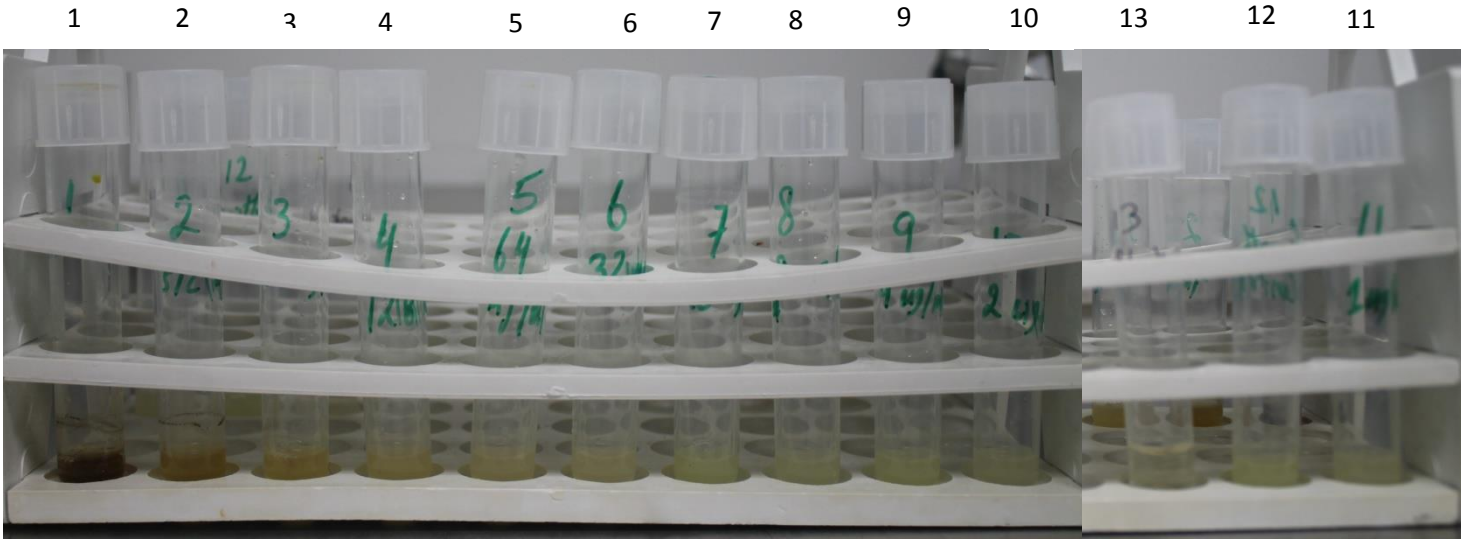


Figure 5. MIC assay against RK395. This fragment is intense in color at high concentration. Thus, absorbance data was very high for higher concentrations of the fragment.

Despite having a high absorbance value, the tube assayed with 1.024 mg/mL of RK395 has significantly lower turbidity than other concentrations. Thus, the MIC value for RK395 against *P.aeruginosa* may lie between 1.0 mg/ml and 1.5 mg/ml

MTT Assay

The 4th generation H-1975 cells growing in plates were treated with trypsin. The mixture was centrifuged at 150g for 20 min at 20°C, and a hemocytometer was used to determine the total number of cells in the falcon tube (using an online hemocytometer calculator). Roughly 16 million cells were determined to be in the tube. Once the cells were done centrifuging, the cell pellet was diluted in 4 ml of media, resulting in a concentration of roughly 4 million cells per ml of media. It was then necessary to calculate the volume of solution from the falcon tube needed to dilute the cells to 5,000 cells per mL in 10 mL of media (0.125 mL). 100 µL aliquots of the diluted cell mixture were pipetted into 92 wells of a 96 well plate (the other four wells were used as blanks containing only media). The following day, 11 different concentrations of DMSO (0.5%, 1%, 2%, 3%.....10%) were added to each column of wells (the 12th column did not contain any DMSO). The cells were incubated overnight. Roughly 12-18 hours later MTT reagent was added to each well, and the plate was incubated for 4 hours. Detergent was then added to each well, and the plate was incubated overnight. Roughly 18 hours later, absorbance values of each well were determined at 540 nm.

% DMSO	Blank	0	0.5	1	2	3	4	5	6	7	8	9	10
Average Absorbance	0.043	0.508	0.381	0.363	0.2175	0.201	0.123	0.095	0.087	0.098	0.092	0.086	0.073
subtraction of blank abs.	0	0.464	0.338	0.320	0.1743	0.157	0.080	0.052	0.043	0.055	0.048	0.043	0.030

Table 6. Averaged absorbance values obtained after a MTT assay with varying concentrations of DMSO

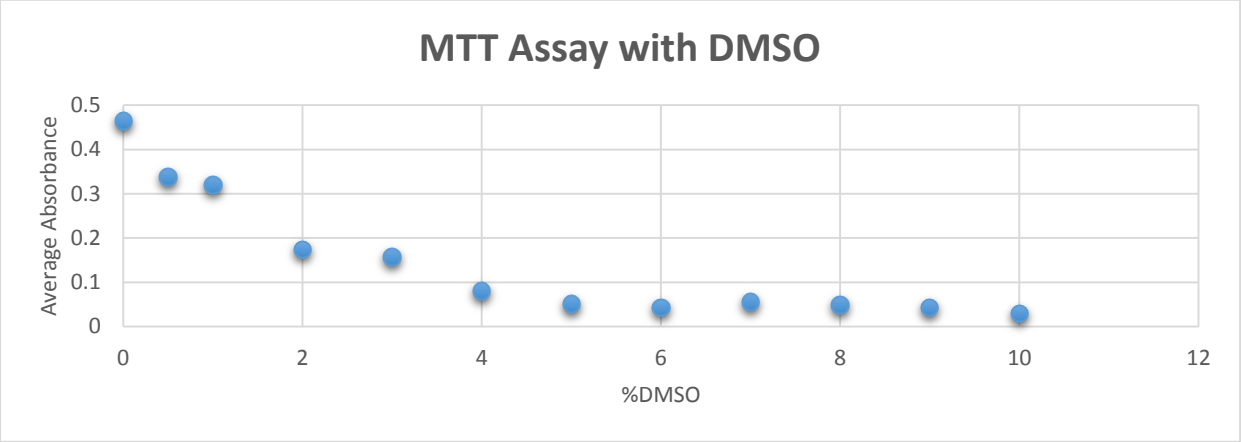


Figure 6.Varying concentrations of DMSO assayed against H-1975 cells. After 1% DMSO, cell viability dropped considerably.

[RK395] (mg/ml)	0	0.002	0.004	0.008	0.016	0.032	0.064	0.0128	0.256	0.512	1.024	2.048
Avg. Absorbance	0.0753	0.075	0.146	0.107	0.105	0.022	0.046	0.083	0.236	0.597	1.23	2.33

Table 7. Averaged O.D. values for MTT assay with RK395

Roughly 12 million cells were isolated from cell culture plates (1.56 million cells per mL). The cells were centrifuged, and the cell pellet was diluted in 4 ml of media, resulting in a concentration of roughly 3 million cells per ml of media. The volume of solution from the falcon tube needed to dilute the cells to 5,000 cells per mL in 10 mL of media was 0.167 mL. 100 µL aliquots of the diluted cell mixture were pipetted into 92 wells of a 96 well plate (the other four wells were used as blanks containing only media). Twelve to 18 hours later, 9 different concentrations of the drug fragment RK395 (0.002, 0.004, 0.008, 0.016, 0.032, 0.064, 0.0128, 0.256 0.512, 1.204, and 6.144 mg/mL) were added to 9 of the 12 columns in the plate (one of the columns was used as a blank and the last one did not contain any drug). The cells were incubated overnight. Roughly 18 hours later, MTT reagent was added to each well, and the plate

was incubated for 4 hours. Detergent was then added to each well, and the plate was incubated overnight at room temperature. Eighteen hours later, absorbance values of each well were determined at 540 nm.

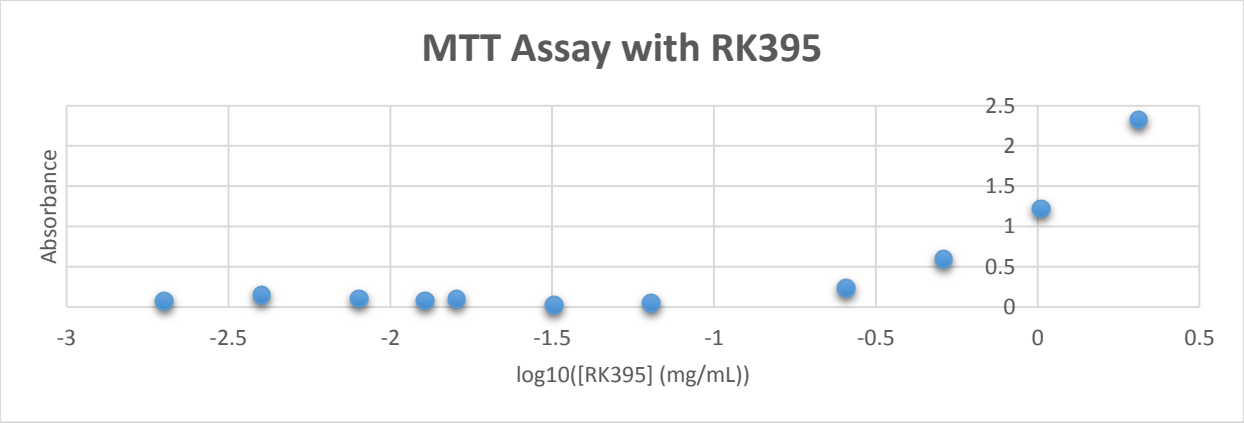


Figure 7. MTT assay with RK395. Because RK395 is intense in color atrelatively high concentrations, an unexpected trend is observed in the graph.

The next assay was done against RK207.Using a hemocytometer calculator, it was determined that roughly 15 million cells were contained within a falcon tube of trypsin treated cells (1.9 million cells per mL). The cells were diluted to 5,000 cells per mL in 10 mL of media. 100 µL aliquots of the diluted cell mixture were pipetted into 92 wells of a 96 well plate (the other four wells were used as blanks containing only media). The following day, 11 different concentrations of the lead fragment RK207 (0.195, .391, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 mM) were added to 11 of the 12 columns in the plate (The first column contained 4 blank wells with just 150 µL of media, and the last three contained 150 µL of cells in media without the drug fragment). The cells were incubated overnight. Twelve to 18 hours later MTT reagent was added to each well, and the plate was incubated for 4 hrs. Detergent was then added to each well, absorbance values of each well were determined at 540 nm.

[RK207] (mg/ml)	0	0.000365	0.00073	0.00146	0.00292	0.005841	0.011682	0.023364	0.046727	0.093454	0.186908	0.373816
Avg. Absorbance	0.65175	0.424	0.382	0.323	0.314	0.321	0.299	0.327	0.301	0.302	0.367	0.233

Table 8. Averaged O.D. values for MTT assay with RK20

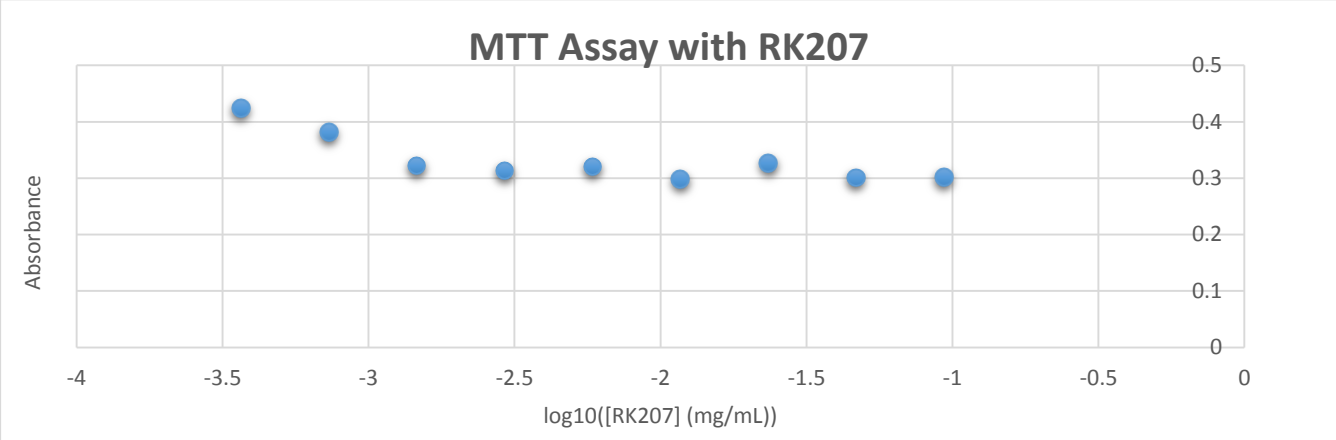


Figure 8. MTT assay with RK207

The graph above represents an MTT assay with RK207. Due to the fact that the 200 and 100 mM concentration data points were outliers, they are not included in the graph. As expected, cell viability decreased with increasing concentration of fragment compound.

RK395 was assayed once more, using smaller concentrations of the fragment. Using a hemocytometer calculator, it was determined that roughly 1.8 million cells were contained within a falcon tube of trypsin treated cells (235,000 cells per mL). The cells were diluted to 5,000 cells per mL in 10 mL of media. 100 µL aliquots of the diluted cell mixture were pipetted into 92 wells of a 96 well plate (the other four wells were used as blanks containing only media). The following day, 11 different concentrations of the lead fragment RK395 (0.0001, .0003, 0.001,

0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 mM) were added to 11 of the 12 columns in the plate (The first column contained 4 blank wells with just 150 µL of media, and the last three contained 150 µL of cells in media without the drug fragment). The cells were incubated overnight. Twelve to 18 hours later, MTT reagent was added to each well, and the plate was incubated for 4 hrs. Detergent was then added to each well, absorbance values of each well were determined at 540 nm.

[RK207] (mg/ml)	0	$1.9X10^{-05}$	$5.74X10^{-05}$	0.000191	0.000574	0.001913	0.005738	0.019127	0.057381	0.19127	0.57381	1.9127
Avg. Absorbance	0.4985	0.42	0.35	0.42	0.43	0.37	0.40	0.25	0.19	0.11	0.13	0.67

Table 9. Averaged O.D. values.

The Graph below represents an MTT assay with RK395. Because the 10 mM concentration data point was an outlier, it is not included in the graph. As expected, cell viability decreased with increasing concentration of the fragment (there were a few exceptions).

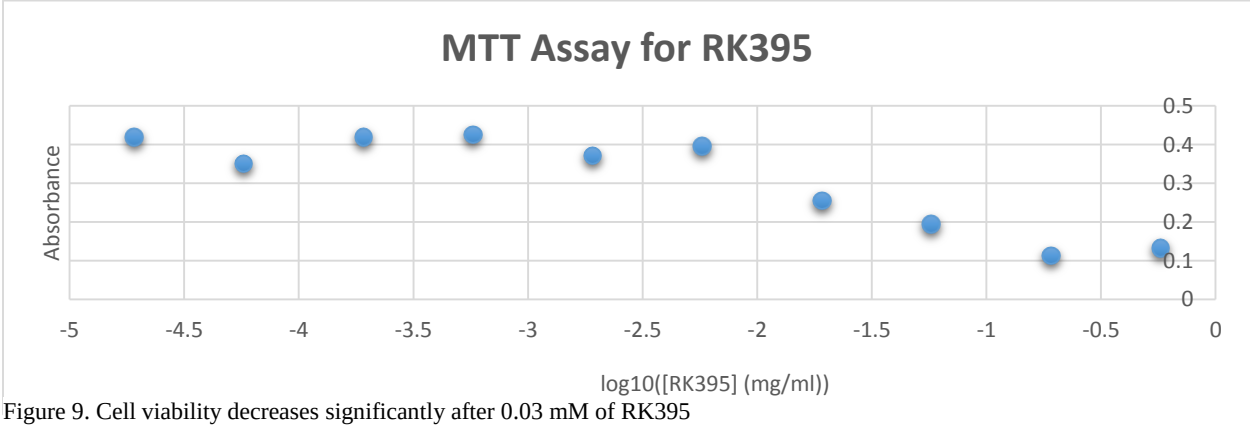


Figure 9. Cell viability decreases significantly after 0.03 mM of RK395

ACP was assayed next. Using a hemocytometer calculator, it was determined that roughly 18 million cells were contained within a falcon tube of trypsin treated cells (2 million cells per mL). The cells were diluted to 5,000 cells per mL in 10 mL of media. 100 µL aliquots of the diluted cell mixture were pipetted into 92 wells of a 96 well plate (the other four wells were used as blanks containing only media). The following day, 11 different concentrations of the lead fragment RK395 acrylic acid product (ACP) (0.00001, .00003, 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1 mg/ml) were added to 11 of the 12 columns in the plate (The first column contained 4 blank wells with just 150 µL of media, and the last three contained 150 µL of cells in media without the lead fragment). The cells were incubated overnight. Twelve to 18 hours later, MTT reagent was added to each well, and the plate was incubated for 4 hrs. Detergent was then added to each well, absorbance values of each well were determined at 540 nm.

[RK207] (mg/ml)	0	0.00001	0.00003	0.0001	0.0003	0.001	0.003	0.01	0.03	0.1	0.3	1
Avg. Absorbance	0.12	0.104	0.103	0.11	0.105	0.123	0.102	0.104	0.12	0.12	0.22	0.58

Table 6. Averaged O.D values

The Graphs below represent MTT assays with RK395 ACP. Due to the fact that the 1 and 0.3 mg/ml concentration data points were outliers, they were not included in any of the graphs (RK395 at these concentrations has a dark color, and skewed the spec data). During this assay, RPMI-1640 media containing 1% Pen Strep was used. This may have influenced the unexpected data collected; it's possible

that there were not enough cells in the wells to produce expected result.

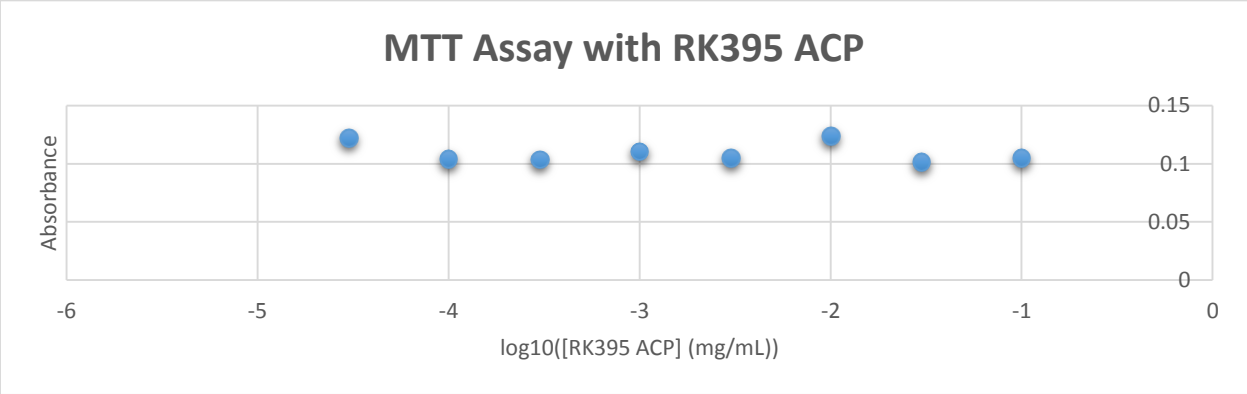


Figure 10. MTT assay with RK395 ACP. Cell viability does not significantly decrease within this concentration range of the fragment compound

NMR

Fragment binders were diverse with a 23% hit rate for the screened library. Cyclohexyl benzene and cyclohexyl methyl benzene scaffolds were the most preferred binding motifs for all three proteins. The hits obtained were weak binders but have the potential to be modified (by adding on additional chemical components) to increase binding affinity (or decrease K_d values).

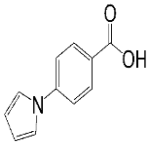
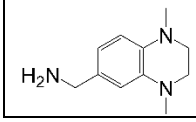
Structure	Fragment	Selectivity	BrmGRX $K_d \pm$ S.D. (mM)	hGRX1 $K_d \pm$ S.D. (mM)	PaGRX $K_d \pm$ S.D. (mM)
	RK207*	BrmGRX	0.98±0.25	2.45±0.52	1.82±0.49
	RK395**	PaGRX	5.49±3.22	1.86±1.15	0.51±0.37

Table 7. Extracted from a table produced by Ram Khattri highlighting dissociation constants for best fragment hits. Selective binding for BrmGRX and PaGRX is observed with RK207 and RK395 respectively

Discussion

To accomplish the ultimate goal of identifying potential lead compounds that bind (and ideally inhibit the activity of) bacterial orthologues of GRX with limited binding to hGRX (i.e. without harming the human host), FBDD was employed using STD NMR.

^{15}N labeled hGRX1, PaGRX and BrmGRX proteins had to be expressed and purified. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) was utilized to confirm the identity of each protein during purification based on their known sizes. NMR samples were prepared to analyze each protein's individual properties, and inspect how they interact with the library of small fragment molecules of potential medicinal significance. Selected hits were isolated and assayed against both bacterial (MIC) and human (MTT assay) cells.

Candidate fragments were either stored in their solid state, or dissolved in DMSO. DMSO was chosen because fragments had to be dissolved in a solvent capable of solubilizing nonpolar compounds (most hits contained nonpolar aromatic substituents), but also capable of forming a homogenous mixture with a polar solvent such as water. Thus, a MTT assay was performed with varying concentrations of DMSO (between 0.5 and 10%) to determine how much DMSO could be added to the cancerous H-1975 cells without significantly reducing their viability. It was discovered that no more than1% DMSO (of the total volume of media, cells, and dissolved lead fragment) could be used in an assay. This was taken into consideration for subsequent assays. RK395 was assayed in the next MTT test; however the data obtained does not support the expected result; cell viability did not decrease with increasing drug concentration. However, the columns with the greatest concentrations of drug were darker in color than the others (hence why the spec data obtained for those wells is higher). Therefore, an MTT assay with RK395 was performed again, at much lower concentrations than were previously tested.

Based on the results from the 2nd assay with RK395, cell viability dropped considerably at concentrations of RK395 above 0.005738 mg/mL. MIC results against RK395 suggest that between 1 mg/mL and 1.5 mg/mL of RK395 is needed to inhibit *P. aeruginosa* grown in liquid culture. Therefore, it was necessary to modify the fragment in order to increase its binding affinity for PaGRX over hGRX. Thus, RK395 acrylic acid coupled fragment was synthesized and assayed with H-1975 cells, at varying concentrations. However, cell viability did not decrease significantly with increase of fragment concentration. An additional assay should be performed at higher concentrations of RK395 ACP.

Like RK395, RK207 was a selective binder to BrmGRX. Based on MTT results with RK207, no more than 0.000365 mg/mL of this compound can be used to treat human cells infected with *B. melitensis*. However, RK207 in association with ruthenium compounds ($RuCl_3$ and Ru-cymene) can increase its binding affinity; potentially allowing for higher concentrations of the modified compound to be used against human cells. The use of transition metals in the creation of organometallic compounds to treat illnesses, such as cancer, is a well-studied area of research. The stereochemical diversity of metals allows them to interact with a number of biological molecules. Positively charged metals can bind negatively charged intracellular targets like nucleic acid and proteins. Ruthenium (II/III) complexes in particular are known anticancer agents. However, they also have antimicrobial properties that can be exploited to potentially inhibit the function of bacterial proteins [14]. Ruthenium complexes in association with potential medicinal fragments, such as RK207, can be guided to the appropriate protein target of a pathogenic bacterial species (such as BrmGRX) and increase the binding affinity of the fragment for the orthologous bacterial protein.

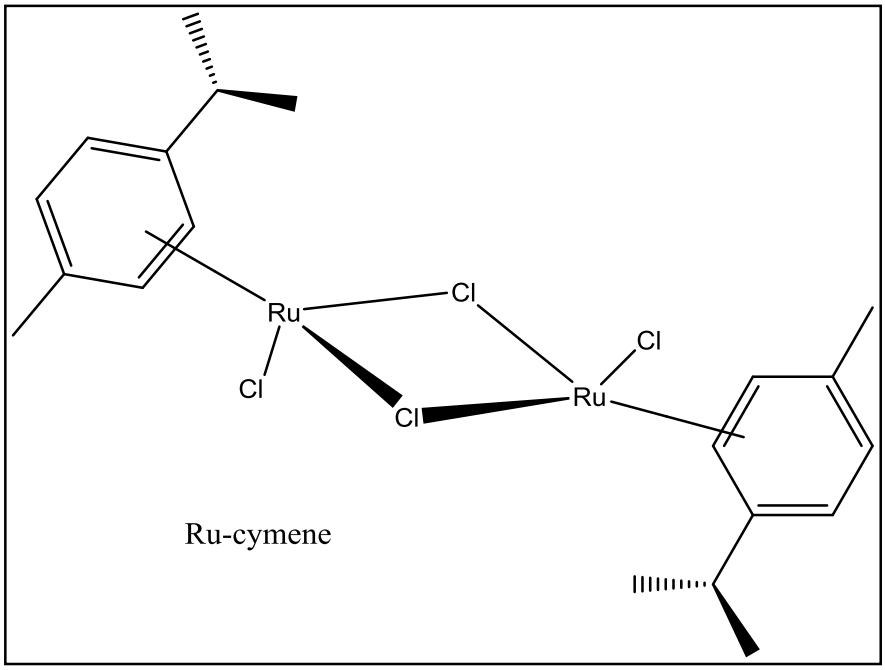


Figure 11. Drawing of dichloro (*p*-cymene) ruthenium (II) dimer (Ru-cymene) using ChemDraw software.

Based on MIC data, Ru-cymene in particular has a MIC of 0.7 mg/mL for *E. coli* and 1 mg/mL MIC for *P. aeruginosa*. This compound (RK207 in association with Ru-cymene) should be assayed with human cells (particularly from tissue that *B. melitensis* tend to infect) to determine its effect on host cells.

By performing MTT and MIC assays on fragment leads, one can determine the therapeutic range—a comparison of the amount of RK207 or RK395 that will produce the desired therapeutic result, to the amount that will result in death of the microorganism— or dosage range, that is large enough (ideally several orders of magnitude) to achieve desired therapeutic results for a large and diverse population of people. A larger therapeutic range increases the chance that the therapeutic agent (RK207 or RK395) will successfully produce desired results. Having a narrow dosage range increases the chances of toxicity, and will require careful monitoring of other variables that may inhibit a cell's ability to functional optimally, such

as dehydration. Therefore, the success of our research largely depends on the therapeutic range of the drugs being tested.

Conclusion

Glutaredoxin proteins are essential components of cellular function and viability. They catalyze a variety of reactions some of which are responsible for synthesizing DNA and ensuring proper immune function. Orthologues of GRX in other organisms, such as bacteria, have similar catalytic function to hGRX. Therefore orthologous proteins of GRX from microbial cells can be selectively targeted to inhibit and/or reduce cell viability. This process is achieved using FBDD STD NMR; by screening a large library of potential candidate fragments capable of binding to bacterial orthologues of GRX, PaGRX and BrmGRX, with limited or no binding to the host or hGRX. Once viable fragments hits are identified, their dosage range (amount needed to kill bacterial cells without killing off human cells) can be determined based on MTT and MIC data. Based on the result obtained in this study, RK207 and RK395 were selective binders of BrmGRX and PaGRX respectively. To increase their antimicrobial activity, it is necessary that they be coupled with other chemical compounds that could potentially increase their binding affinity for the target microbial GRX.

As a result of working on this project, I have acquired invaluable knowledge of biochemical topics and techniques. While I have enhanced basic lab skills including, bacterial transformation, protein expression and purification, SDS-PAGE, and eukaryotic cell culturing; I've also learned how to think critically about results in order to make appropriate manipulations to a procedure that may produce more ideal results. I learned to interpret MIC and MTT data in

order to make necessary changes in concentration of fragments assayed against bacterial and human cells in subsequent test.

Acknowledgments

I would like to thank the graduate students, including Ram Khattri, Dan Morris, Caroline Davis, and Stephanie Bilinovich for providing me with guidance and resources to carry-out my experiments. The protein purification skills I learned and enhanced are partially attributed to my experience working with Ram Khattri, who initially provided step-by-step guidance of necessary procedures and proper use of protein purification equipment. Many of the protein samples purified were used for NMR screening conducted by Ram. Ram is also responsible for demonstrating how to conduct a MIC assay. The lead fragments used for MTT assays were provided by both Ram and Caroline. Dan Morris provided guidance on proper MTT assay procedures. I would also like to thank the Buchtel College of Arts and Sciences for funding our research, as well as my project sponsor, Dr. Thomas Leeper, for his guidance.

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Appendix

General Equipment Care and Safety

During bacterial transformation, cells were transferred using proper aseptic techniques; including, using a Bunsen burner to sanitize the wire loop the cells were transferred with. Gloves and goggles were worn at all times. When culturing eukaryotic cells, and performing MTT assays, the laminar flow hood, and inverted microscope were both wiped with 70% EtOH before and after being used. The tube connected to the vacuum pump used to remove cells, was cleaned with 10% bleach after each use. MTT is very toxic; special care was taken not to infect the skin or eyes with this compound. The detergent reagent is an irritant, and should be washed off immediately if it contacts the skin or eyes. Care was taken not to contaminate any of the MTT reagents with the cancer cells (MTT that is a blue or green color indicates contamination).

L-B Broth Recipe:

- 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter autoclaved at 121 on liquid cycle for 15 minutes

M9 Recipe

	1 L
Dissolve First:	
Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
Then add one at a time:	
40mg/mL Thiamine-HCl(*filter sterilized*)	1mL
1 M $MgSO_4$(*autoclaved*)	1.0 mL
1 M $CaCl_2$(*autoclaved*)	0.2 mL

Antibiotic	
Trace Metals (see below)	10 mL
Adjust to final volume, filter sterilize, then add:	
NH_4Cl (^{15}N when applicable)	0.5 g
D-glucose (^{13}C when applicable)	2.0 g

Trace Metals Stock (100X) :

1. Dissolve 5 g/L $Na_2EDTA{\cdot}2H_2O$ in 800 mL ultrapure H_2O and then correct to pH 7.0 when completely in solution
1. Add one at a time, letting each fully dissolve:
   0.83 g $FeCl_3{\cdot}6H_2O$
   0.05 g $ZnCl_2$
   0.01 g $CoCl_2{\cdot}6H_2O$
   0.013 g $CuCl_2{\cdot}2H_2O$
   0.01 g H_3BO_3
   1.60 g $MnCl_2{\cdot}6H_2O$
1. Correct volume to 1 L and autoclave solution (may be stored at room temp or 4°C)

Buffer A and B

Buffer A:	Buffer B:
40 mM Na-Phos pH 7.0 24.2 mL 0.5M dibasic 15.6 mL 0.5M monobasic	40 mM Na-Phos pH 7.0 24.2 mL 0.5M dibasic 15.6 mL 0.5M monobasic
200 mM NaCl- 20 mL 5M stock	200 mM NaCl- 20 mL 5M stock
2mM DTT- 1mL 1M stock	2mM DTT- 1mL 1M stock
20 mM imidazole- 0.681g If no DTT available use 0.2 mM TCEP	400 mM imidazole- 13.616g