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Identification of synthetic chemical compound inhibitors of Pseudomonas aeruginosa protein synthesis and the development of lead series

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IDENTIFICATION OF SYNTHETIC CHEMICAL COMPOUND INHIBITORS OF PSEUDOMONAS AERUGINOSA PROTEIN SYNTHESIS AND THE DEVELOPMENT OF LEAD SERIES

A Thesis

by

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ABSTRACT

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Pseudomonas aeruginosa is a ubiquitous Gram-negative bacteria and a primary cause of nosocomial infections. An aminoacylation/translation (A/T) assay was developed using *P. aeruginosa* components to perform poly(U) mRNA directed protein synthesis. Using scintillation proximity assay (SPA) technology a high-throughput screening (HTS) platform was developed. A synthetic chemical compound library (>900) was screened, 36 hit compounds were identified and molecular targets were determined. Compounds were analyzed for enzymatic inhibition (IC_{50}) and inhibition of bacterial cultures (MIC). Time-kill studies determined bacterial growth to be bacteriostatic or bactericidal in the presence of inhibitor. Mechanism of action, effects on eukaryotic cytosolic and mitochondrial protein synthesis were determined. Cytotoxicity using a mammalian cell line and global mode of inhibition was determined. One compound was evaluated for the ability to generate spontaneous resistant mutants or develop resistance after serial passage.

DEDICATION

The completion of master studies would not have been possible without the love and support of my family. My mother, Alma Rosa Alvarez, my husband, Fernando Galvan, and my daughter, Sofia Fernanda Galvan and lastly my my in-laws; including my father in law Fernando Galvan my mother in law Nancy Galvan Martinez and my two sisters in law, Nancy Madahi and Edna Ivette Galvan. They all have motivated and supported me by all means in accomplishing this degree. Thank you for all your help, love and patience.

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great hearts and are responsible for my academic endeavors.

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CHAPTER I

BACKGROUND

A Gram-negative microorganism, *Pseudomonas aeruginosa*, is an opportunistic bacterial pathogen and a causative agent in a wide range of infections. Infections range from bacteremia and urinary tract infections to burn wound infections and pulmonary infections; *P. aeruginosa* is ubiquitous in nature. In the hospital setting, *P. aeruginosa* is responsible for approximately one-seventh of all infections, and has been identified as the most common Gram-negative pathogen located in the intensive care unit (1,2). A particular serious medical problem caused by *P. aeruginosa* is lung infections associated with cystic fibrosis, from which chronic infections are the main causes of patient morbidity and mortality (3, 4). Clinical isolates of antibiotic resistance strains of *P. aeruginosa* are significant and growing; with multidrug-resistant strains becoming increasingly common in hospital patients (5,6).

On a global scale, antibiotic resistance has become a serious problem due to an increase in percent of incidences as well as the lack of innovative antibiotics available. The number of new antibacterial agents approved by the FDA has dramatically decreased since 1983 (7) (Figure 1). Of the new antibiotics that have been approved, most are derived and are modified forms of previously marketed antibiotics. Although this has been a successful short-term strategy, it is not fix for a growing worldwide health issue.

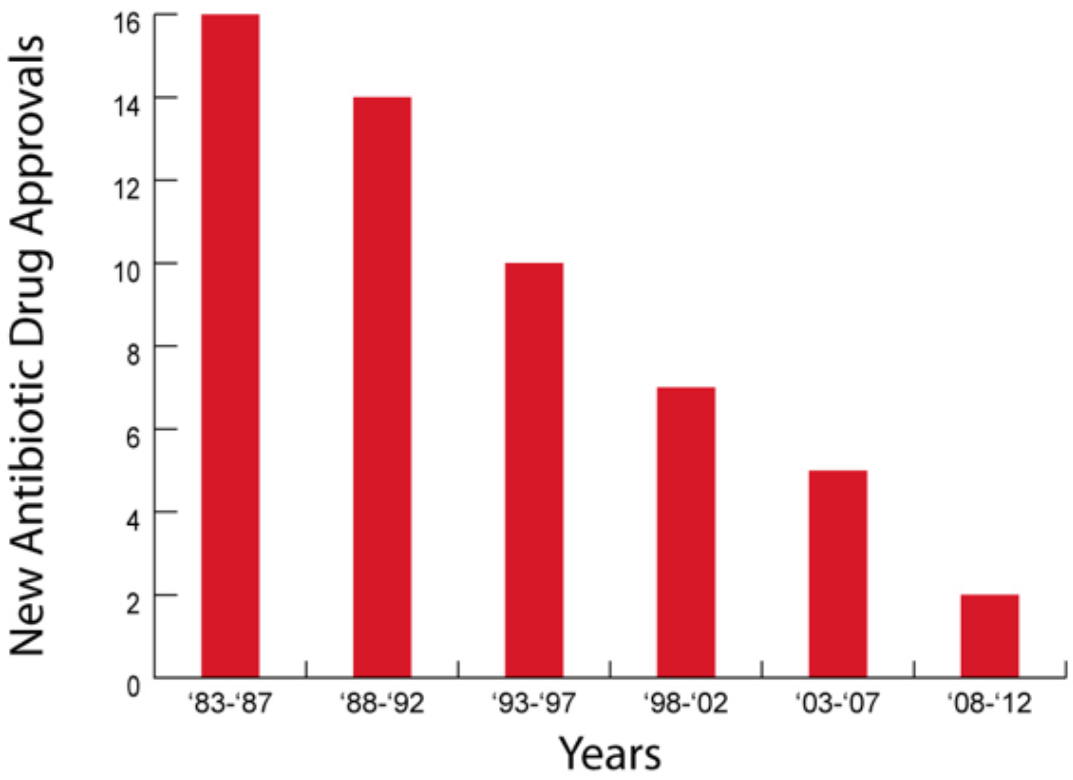

Figure 1. New Molecular Entity (NME) Systemic Antibiotics Approved by the US FDA since 1983 (7).

Protein synthesis is a metabolic process that is fundamental in both the biological and microbiological systems. This metabolic pathway provides proteins to carry out cellular functions. Protein synthesis is the process by which amino acids are linearly arranged into proteins through the involvement of ribosome, transfer RNA (tRNA), messenger RNA (mRNA), and various enzymes. Protein synthesis is also called translation, the process of gene expression. The central dogma of molecular biology states that deoxyribonucleic acid (DNA) encodes ribonucleic acid (RNA) through the process of transcription, which in turn, RNA expresses proteins through the process of translation (8).

The translational process of protein biosynthesis has three major steps: initiation, elongation, and termination. In the initiation step, initiation factors 1, 2 and 3 (IF1, IF2, and IF3)

function in the formation of the initiation complex. This complex contains mRNA attached to a functional 70S ribosome with the start codon positioned at the P-site, and the initiator tRNA bound at the P-site by codon-anticodon pairing with the mRNA. The elongation step, in addition to the ribosome, requires three enzymatic components: elongation factor Tu (EF-Tu) elongation factor Ts (EF-Ts) and elongation factor G (EF-G) for the attachment of one amino acid to a growing peptide chain per cycle. Finally, the termination step requires a stop codon on the DNA to be located in the A-site of the ribosome which is recognized by one of the three ribosomal release factor: RF1, RF2 and RF3. The newly formed peptide is released from the ribosome; ribosomal components disengaged and new cycle is ready to begin with the help of EF-G (9).

The protein synthesis system in this project is different from that of the biological system in that it only contains the elongation step. This step is directed by poly(U) mRNA and requires the aminoacylation of tRNA by phenylalanyl-tRNA synthetase (PheRS) to construct a polypeptide to code for phenylalanine protein, therefore this is also called the Aminoacylation/Translation (A/T) system. This system is composed of enzymatic and non-enzymatic components. The enzymatic components include: the ribosome, which is the universal cellular organelle that translates the genetic code that composes the mRNA by sequential polymerization of amino acids to form functional proteins (10); elongation factor Tu (EF-Tu), which delivers the aminoacylated tRNA (aa-tRNA) to the A-site of the ribosome in a ternary complex with a guanosine-5'-triphosphate (GTP). The ternary complex binds the A-site of an actively translating ribosome in a mRNA-dependent manner. Once the cognate ternary complex is bound to the ribosome and the aminoacylated tRNA is delivered to the ribosome, GTP is hydrolyzed to guanosine diphosphate (GDP) by the activation of the GTPase actibity of EF-Tu. The resulting EF-Tu•GDP complex dissociates from the ribosome and is recycled to the active

EF-Tu•GTP complex in a nucleotide exchange reaction catalyzed by elongation factor Ts (EF-Ts) (11, 12, 13). Another enzymatic component, EF-G is involved in two distinct steps: elongation and ribosome recycling. During the elongation step, EF-G binds the ribosome and promotes the movement of tRNA and mRNA relative to the ribosome (14, 15). The relative shift of the mRNA is by a distance of one codon and the peptidyl- and deacylated-tRNAs are shifted from the pre-translocational to the post-translocational sites (16). During the recycling step, EF-G acts in concert with the ribosome-recycling factor (RRF) to effect the disassociation of the ribosome into its individual subunits (17). We have cloned and over expressed two forms of EF-G present in *P. aeruginosa* of which, proteins encoded by these genes are both members of the EF-G 1 subfamily. The gene encoding one of the forms of EF-G is located in the *str* operon and the resulting protein is referred to as EF-G1A while the gene encoding the other forms of EF-G is located in another part of the genome and the resulting protein is referred to as EF-G1B. Finally, an amino acyl-tRNA synthetase (aaRS) is used in the attachment of an amino acid to the cognate tRNA, this process is called the aminoacylation or charging of the tRNA (18). In the A/T system, phenylalanyl-tRNA synthetase (PheRS) was used since the reaction is poly (U) mRNA driven.

The non-enzymatic components for the system include: tRNA, which serves as the physical link between the nucleotide sequences of the mRNA encoding for a specific amino acid to be inserted into a growing polypeptide chain (19). Energy providers for the system included ATP and GTP. Phosphoenolypyruvate (PEP) and pyruvate kinase (PK) form an energy source regeneration system in order to maintain constant levels of ATP and GTP (20). Mg^{2+} functions as a cofactor in various steps of the system and K^{+} is needed to stabilize several proteins. Dithiothreitol (DTT) is used to stabilized enzymes in the system by creating a reducing

environment and preventing disulfide bond formation. Poly(U) is used as the mRNA which encode for phenylalanine thus creating a poly-Phe peptide. To monitor the production of the poly Phe produced, [^{3}H] (tritium) labeled phenylalanine is used to detect a radioactive signal.

Antibacterial agents interfere with cellular processes that are essential for the survival of the cell. For both naturally occurring and synthetic antibiotics, the ribosome and protein synthesis in general, are a major target of antibiotic action (21) (Figure 2). Resistance has been detected in almost all these antibiotics shown and in some bacteria; resistance has been acquired to more than one or even all of the antibacterial currently in use. On average, it takes 8 years from the time of introduction of an antibacterial agent on the market until the first incident of bacterial resistance is reported (12) (Figure 3). In order to keep-up with the pace of resistance, there is a critical demand for the development of new novel antibiotics that have different mechanisms of action.

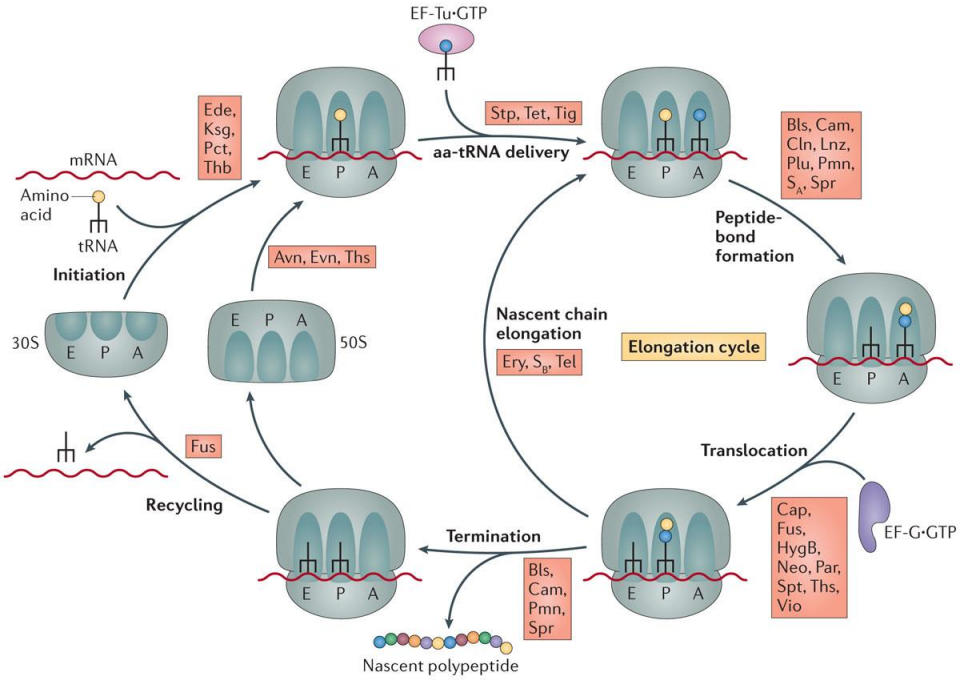

Figure 2. Antibiotic target sites during bacterial protein synthesis (21).

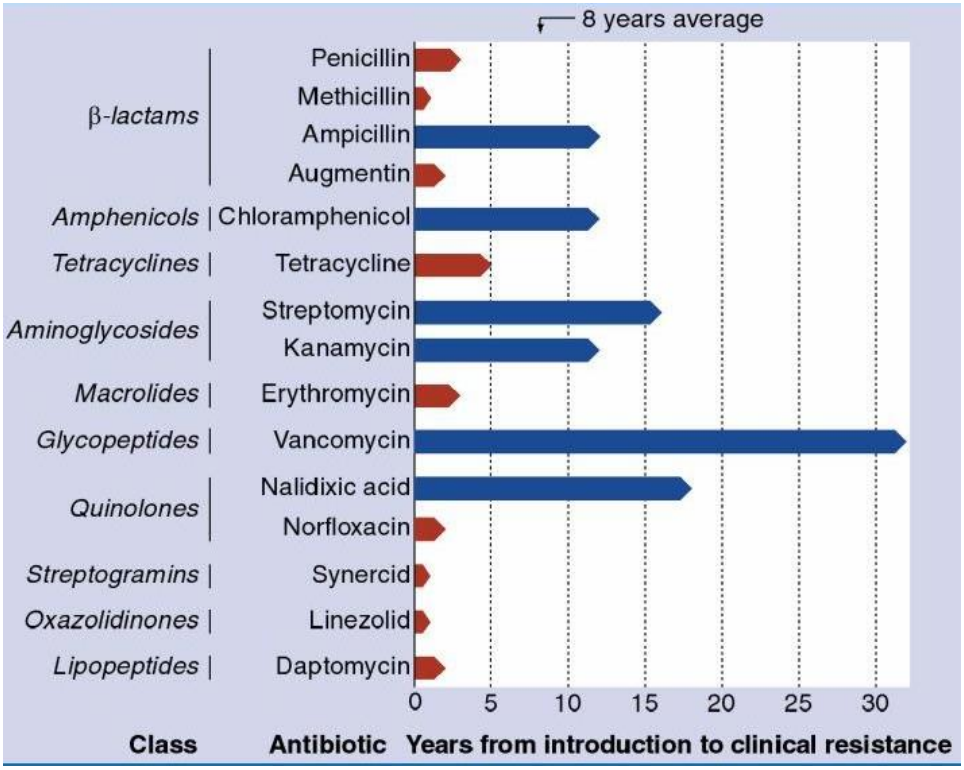

Figure 3. Evolution of antibiotic resistance for several classes of antibiotics (12).

The goal of this project is to use a screening platform based on the A/T system of protein synthesis from *P. aeruginosa* to screen for inhibitors of the system to identify compounds that may be developed into new antibiotics. This includes: 1.) Using an optimized protein synthesis system to screen a synthetic chemical compound library; 2.) Determination of antibacterial activity of the inhibitor; 3.) Study the effects the inhibitor has in a eukaryotic system; 4.) Study the effects that bacteria goes through when acquiring resistance; and 5.) Develop a lead series of chemical compounds that could potentially increase potency of inhibition.

CHAPTER II

LITERATURE REVIEW

Antimicrobial Drug Resistance

Antibiotic/Antimicrobial resistance is the ability of microbes to resists the effects of drugs, that is, bacteria are not killed or their growth is not impaired. Emergence of resistance to one or multiple antimicrobial agents in pathogenic bacteria is spreading throughout the world and has become a serious problem that significantly threatens public health for various reasons. Antibiotic resistance was initially problematic in a hospital setting, this has now extended into the community causing severe infections that are difficult to diagnose and treat. Adding to this problem, research on the development of new anti-infective agents has slowed down considerably since 1962. This has resulted in what is termed the "innovation gap," which covers the period from the time quinolones and streptogramin were first applied to anti-infective therapy until 2000, when the oxazolinidones were introduced (22, 23). This disengagement of antibiotic research is mainly non-scientific, but rather purely an economical phenomena (24, 25). Nevertheless, multiple drug resistance (MDR) bacteria are on the rise to which the human population is at fault. In order to keep pace with emerging resistant pathogens, understanding the changes visible in the molecular level of a cell, allows for the development of new approaches in managing the infections and to create new strategies in the development of new treatments against these bacteria.

Mechanism of Drug Resistance

To be fit for survival, all living organisms strive to adapt to their environment. It is no surprise that bacteria have shown a remarkable ability to endure and adapt to their environment, including the development of different mechanisms of resistance antimicrobial agents. Therefore, many strains of bacteria have become resistant to the therapeutic agents, and thus these drugs rendered ineffective as treatments of choice for severe infection caused by the pathogens.

Bacterial resistance starts at the genetic level, in that the genetic makeup of the previously susceptible bacteria changes via mutation or by the uptake of new genetic information (26). Expression of genes containing the genetic changes in a bacterial cell cause changes in the biological mechanisms that determine the specific type of resistance that the bacteria develop, resulting in a myriad of possible biological forms of resistance (27, 28). This result in susceptible bacteria within the population of bacteria will die whereas the resistant strains will survive. These surviving bacteria can now spread the "resistant coding gene" and transfer the gene to other bacteria via conjugation (transmission of plasmids among bacteria), transformation (direct passage and incorporation of free DNA), and transduction (virus containing bacterial resistance gene infects the new bacterial cell) (28).

Whichever method of transferring a gene to a bacterium this results in the development of antibiotic resistance and production of a tangible biological effect resulting in the loss of activity of the antibiotic. Theses biological mechanisms vary from a reduced permeability or uptake, enhanced efflux, enzymatic inactivation and alteration or over-expression of the drug target (Figure 4) (29).

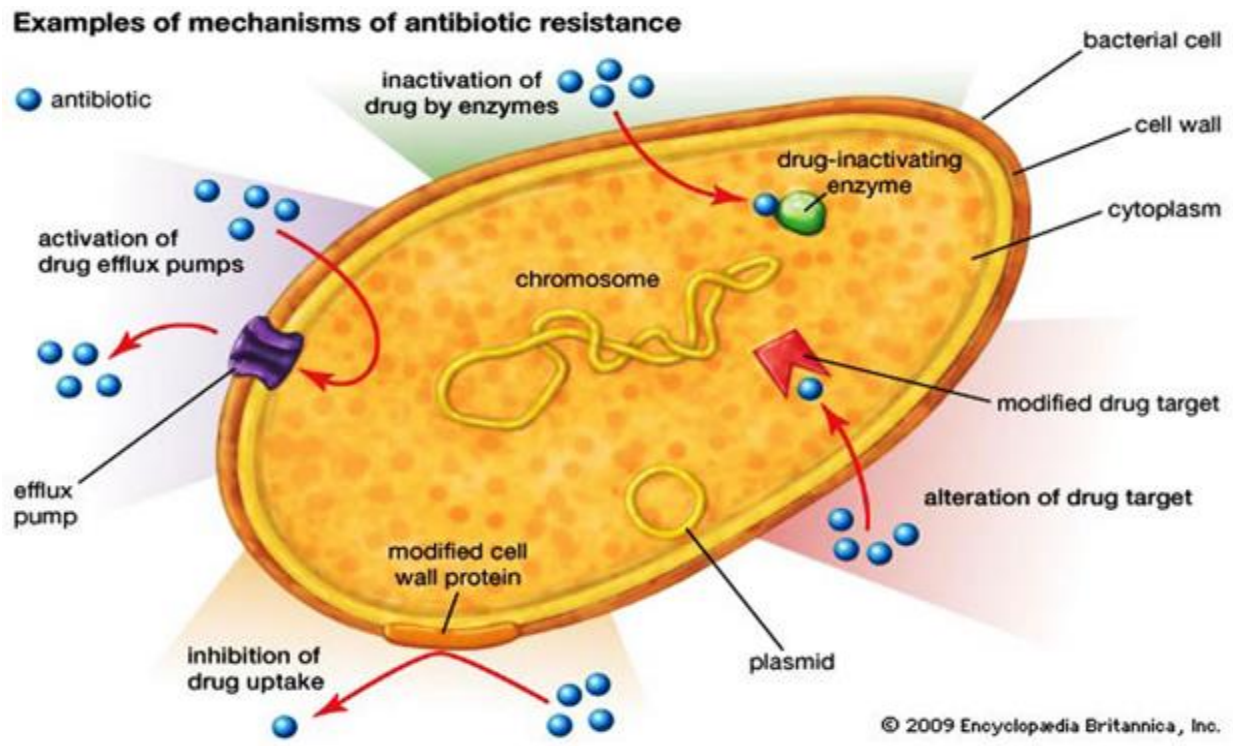

Figure 4. Biological mechanism resulting in antibiotic resistance (29)

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Prior to changes that bacteria go through at the molecular level, one must ask, "What triggers the bacteria to acquire resistance to an antibiotic? The answer to the question revolves around the habits of the human population. Abuse in the use of antibiotics in clinical practice results in selective pressure, allowing bacteria to acquire resistance to an antibiotic. In today's health care system physicians have only a few minutes to fully evaluate a patient, make a diagnosis and prescribed a treatment (30,31). To this, many antibiotics are over-prescribed for the simple act of having a cold, sore throat or ear infections caused by a viral infection. In certain regions, antibiotics are much easier to obtain without a prescription. A definite problem seen in the Rio Grande Valley that has Mexico as the neighboring country, where antibiotics are easily

available without a prescription. Of all the antibiotics produced in the United States, more than half of the antibiotics are used for agricultural purposes (32). Antibiotics are frequently given to animals to increase survival rates in stressful and unsanitary conditions; as well a part of the process used to manufacture food, especially meats (33). Ultimately all these reasons lead to the rise in antibiotic resistance.

Screening Strategies

The widespread and increasing occurrence of antibiotic resistance should not represent an alibi for discontinuing antibiotic research, but should instead represent a stimulus to pursue antibiotic research with renewed energy and enthusiasm. High-throughput screening (HTS) is the process of testing a large number of diverse chemical structures against disease causing targets to identify compounds that inhibit activity of the target (34). Different strategies have been used for identifying novel antibacterial compounds starting from the golden age of antibiotics discovery (1940s-1960s), where whole cell screening of primarily natural compounds led to the identification of most currently known antibiotic classes. This involves microbiological primary screening to identify compounds having antimicrobial activity followed by secondary screening, which is composed of characterization of the initial hit compounds and at the possible development of lead compounds (35). Although very successful in the past, this approach would likely "rediscover" known inhibitors and would also miss a large number of potentially interesting molecules that do not or hardly penetrate the test cells (36, 37). Also, when using whole cell screening, there is a large difference in sensitivity amongst various bacteria that are used (38).

Another approach, which can complement whole cell screening, is the *in-vitro* screening of select targets. This a+pproach offers the advantage of identifying inhibitors having the desired biological activity, independent of their ability to gain access to the interior of the bacterial cell (39). Ideally, *in-vitro* screening of select targets would focus on essential cell function performed by a protein complex or single-proteins with a known 3D structure so that rational design and ligand-based design can be applied to improve the performance of the inhibitor (35). This strategy is the basis this project.

Scintillation Proximity Assay (SPA) Technology

Scintillation proximity assay (SPA) technology is used to detect compounds that inhibit the aminoacylation/translational activity of this project. SPA uses a small scintillant containing microsphere (5 pico-meter in diameter) to bind the target of interest. When a radio-isotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy is transferred from the emitted β-particle, resulting in the emission of light (40, 41). The radioisotopes remaining free in solution will be too distant from the scintillant, and the β-particle will dissipate the energy into the aqueous medium and remain undetected (34, 42).

CHAPTER III

MATERIALS AND METHODS

Aminoacylation/Translation Assay

A scintillation proximity assay (SPA) was developed to monitor inhibition of the aminoacylation/translation (A/T) assay. The optimal concentrations of all enzymatic components were arrived at through sequential rounds of optimization and set at concentrations just below the saturation point of the titration curve to allow monitoring of inhibition of each component. The nonenzymatic components consist of, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 10 mM $MgCl_2$, 0.03 mM spermine, 1 mM dithiothreitol (DTT), 1.5 mM ATP, 0.5 mM GTP, 40 μM [^{3}H]Phenylalanine (75cpm/pmol) and 0.3 mg/mL poly(U)RNA . To maintain constant levels of ATP and GTP in the assays 4 mM phosphoenolpytuvate (PEP) and 0.025 Units/μL pyruvate kinase (PK) were added as a nucleotide regeneration system. The *P. aeruginosa* enzymatic components consists of, 0.2 μM ribosomes, 0.075 μM PheRS, 1.0 μM EF-Tu, 0.05 μM EF-Ts, and 0.2 μM EF-G.

The screening reactions were carried out in 96-well microtiter plates (Costar), consisting of, 33 μL of protein/substrate mix (without tRNA) and 2 μL of chemical compound (3.3 mM) dissolved in 100% DMSO. This mixture was allowed to incubate at ambient temperature for 15 min and initiated by the addition of 15 μL of *E. coli* tRNA. The reaction plates were incubated at 37 °C for 1 hour and stopped by the addition of 5 μL of 0.5 M

ethylenediaminetetraacetic acid (EDTA). 200 μg of SPA beads (RNA Binding Beads (YSI), Perkin Elmer) in 150 μL of 300 mM citrate buffer (pH 6.2) were added. The plates were analyzed using a 1450 Microbeta Jet Liquid Scintillation and Luminescence Counter (Wallac).

To confirm primary hits, assays were as described above in duplicate form for reliable results. To determine IC_{50} values, confirmed hits were serially diluted from 200 μM to 0.4 μM into assays described above.

Target Determination Assays

PheRS Aminoacylation Assay

To determine the effect of the confirmed chemical compounds on the activity of PheRS, assays were carried out as described (43). Exception was the enzyme mix was pre-incubated with 132 μM of chemical compound for 15 min prior to the addition of tRNA and 1 hr incubation after addition of tRNA at 37 °C. Reactions were stopped by the addition of 5 μL of 0.5 M EDTA. 400 μg of SPA beads (poly-lysine PEI-PVT Beads, Perkin Elmer) in 150 μL of 300 mM citrate buffer (pH 2.0) were added and plates were analyzed as described above.

EF-Tu and EF-Ts GDP Exchange Assay

Nitrocellulose binding assays were used to determine the effect confirmed compounds on GDP exchange by EF-Tu as previously described (44) with the exception that the enzyme mix was pre-incubated with 132 μM of chemical compound for 15 min prior to the addition of [^{3}H]GDP.

EF-G GTPase Assay

Assays to determine the effect of confirmed compounds on ribosome-dependent GTP hydrolysis by EF-G were carried out as previously described (45). Exception was the enzyme mix was incubated with 132 μM of test compounds. Reactions were at 37 °C for 30 min and the assays were stopped by the addition of 150 μL of 50 mM EDTA. The amount of GTPase activity was determined by measurement of the amount of P_i liberated using a colorimetric GTPase assay Kit (Novus Biologicals).

LeuRS Aminoacylation Assay

To determine the effect of the chemical compounds on the activity of LeuRS, assays were carried out as described (46). Exception was the enzyme mix was pre-incubated in glass tubes with 132 μM of chemical compound for 15 min prior to the addition of tRNA in 37 °C water bath. Reactions were stopped by the addition of 2 mL ice cold 5% tricholoroacetic acid (TCA), followed by filtration of solution through glass-fiber filter (Whatman). Filters were washed with twice with 2 mL ice cold 5% TCA. Filters were dried and counted using the LS6500 multipurpose scintillation counter (Beckman Coulter, Brea, CA).

Minimum Inhibitory Concentration (MIC) Testing

Broth microdilution MIC testing was performed in 96-well microtiter plates according to the National Committee for Clinical Laboratory Standards (NCCLS) (47). MIC values were determined against 10 pathogenic bacteria: *Enterococcus faecalis* (ATCC 292912), *Escherichia coli* (ATCC 25922), *Haemophilus influenza (ATCC 49766),*

Moraxella catarrhalis (ATCC 25238), *Pseudomonas aeruginosa* (ATCC 47085), *Staphylococcus aureus* (ATCC 29213), and *Streptococcus pneumonia* (ATCC 49619); all these bacterial strains were acquired from the American Type Culture Collection (Manassas, VA). *Pseudomonas aeruginosa Hypersensitive strain* (ATCC 35151), *Pseudomonas aeruginosa PA200 Efflux* (efflux pump mutant), *E. coli TolC Efflux* (efflux pump mutant) were a kind gift from Urs Ochsner (Creston Pharma-Boulder CO). Chemical compound inhibitors were serially diluted from 128 μg/mL to 0.125 μg/mL in 96-well plates (costar), and incubated with the bacteria at an optical density (OD_{625}) of 0.08 to 0.13. This concentration is equivalent to (0.5-1) $\times 10^8$ colony forming units (CFU). Incubation of compound and bacteria was for 18-24 hours at 37 °C and MIC value was reported as the lowest concentration of inhibitor that prevented visible bacterial growth.

Time-kill Study

Time-kill study was performed using *H.influenza* and *S.aureus* according to the NCCLS (48). Growth media were Haemophilus Broth and Trypticase Soy Broth from Remel (Lenexa, KS). For the experiment, 10 mL of broth medium was inoculated with 0.1 mL of fresh overnight growth culture and grown at 37 °C with shaking (200 rpm) for 2-3 hours. Several pre-warmed flasks containing 10 mL of medium alone or 10 mL of medium containing test compounds (BT_04E11, BT_06G06, BT_06D07, and BT_08F11) at 4 × MIC value were then inoculated with 0.1 mL of the exponential growth cultures. Samples were removed at 0, 2, 4, 6, and 24 h, and serial dilutions were plated on blood agar media to allow for colony enumeration and calculation of the live cell density after 24 h.

Binding Mode Assay

To determine compound competition with ATP, IC_{50}'s were determined as described above, but containing the indicated concentration of ATP concentrations (25, 50, 100, 250, 500 and 1000 μM) (49). Reactions contained one of the indicated concentrations of ATP (25, 50, 100, 250, 500 and 1000 μM), 50 mM Tris-HCl (pH 7.5) 8 mM $MgCl_2$, 0.5 mM spermine, 50 μM [^{3}H]Phe (75 cpm/pmol), 1.0 mM DTT, and 0.075 μM *P. aeruginosa* PheRS. 33 μL of the mix was added to 2 μL of compound and allowed to incubate at room temperature for 15 min. Final compound concentrations in the reactions ranged from 200 μM to 0.4 μM. The reaction was started by addition of 15 μL tRNA and incubated at 37 °C for 1 hour. The reaction was stopped by addition of 5 μL of 0.5 M EDTA. 400 μg of SPA beads (poly-lysine PEI-PVT Beads, Perkin Elmer) in 150 μL of 300 mM citrate buffer (pH 2.0) were added and plates were analyzed as described above. This same process was done against the 6 stated ATP concentration.

To determine if the compounds were competitive with Phe, the same assay was used, however, ATP was now held constant at a concentration of 2.5 mM while varying concentration of Phe (25, 50, 100, 200 and 300 μM) was used (50).

Eukaryotic Protein Synthesis Assay

Assays to determine inhibition of the activity of human mitochondrial PheRS (hmPheRS) by chemical compounds were as described (50) with the exception that the enzyme mix was pre-incubated with chemical compound ranging from with 200 μM to 0.4 μM for 15 min prior to the addition of tRNA.

Reactions used to test chemical compound inhibition on eukaryotic protein synthesis

were carried out using wheat germ extract (Promega) as described in (20). All components were assembled on ice, mixed with the compound and incubated at 37 °C water bath for 1 h. Reactions were stopped by the addition of 2 mL of 5% TCA and heated to 90 °C for 15 min. Reactions were filtered using glass-fiber filters (Whatman), dried, and counted using LS6500 multipurpose scintillation counter (Beckman Coulter, Brea, CA). Concentrations of compounds ranged from 200 μM to 0.4 μM.

In-Vitro Cytotoxicity Test

To determine the effects of the hit compounds on the growth of mammalian cell cultures, *in vitro* cytotoxicity testing was carried out using 3T3 cells (50). Eighteen hours prior to the assay, NIH/3T3 cells were plated per well in a 96-well plate using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin Solution. Compounds were diluted in DMSO, yielding a final DMSO concentration of 6.25% in the cell cultures. Cells were grown at 37 °C under 5% CO_2 and treated with the compounds or DMSO alone for 24 h. The Trevigen TACS MTT Cell proliferation Assay Kit (Gaithersburg, MD) was utilized to assess impact on human cell proliferation and/or viability. Ten microliters of MTT reagent was added to each well and incubated under 5% CO_2 at 37 °C for additional 4 h. The optical density (A_{595}) was determined using a Bio-Rad iMark microplate absorbance reader. Samples were carried out in triplicate form and the student two-tiered *t* test was utilized to assess statistical significance.

Macro-Molecular Synthesis (MMS) Assay

To determine the global mode of action of inhibition of the hit compounds on the growth of bacteria, macro-molecular synthesis (MMS) assay was used (51). MMS assays were performed using the radiolabeled precursors [5-^{3}H] uridine, [methyl-^{3}H] thymidine, L-[3-^{3}H] alanine, and L-[4, 5-^{3}H (N)] leucine to determine if RNA, DNA, cell wall or protein synthesis was inhibited. *P aeruginosa* hypersensitive cells were grown statistically for 6 h at 37 °C in TSB broth until OD_{600}>1. Cells were harvest and re-suspended in 0.5 mL of saline solution and dilution needed to match the 0.5 McFarland standard (approximately 10^{8} CFU/mL) was determined. Upon determining dilution factor, 1 mL saline solution at 30 ×. 0.5 McFarland was prepared and used for plating. A 96-well microtiter plate contains 50 μL of serially diluted compound per well with concentrations ranging from 0.4 to 200 μg/mL. The plate was inoculated with 50 μL of bacterial growth that was determined to be 30 x 0.5 McFarland and incubated for 20 min at 37 °C. Radiolabeled precursors were added (10 μL) and incubation was continued for 20 minutes at 37 °C and stopped by the addition of 100 μL of 20% ice-cold TCA. Plates were refrigerated for 1 h to allow cell lysis and precipitation of macromolecules this was followed by vacuum filtration of the samples through 96-well Durapore plates (Millipore, Bedford, MA). The filter plates were washed twice with 200 μL of 10% ice-cold TCA and then once with 200 μL of cold ethanol and air-dried. 100 μL of MicroScint O (Perkin-Elmer, Boston, MA) was added, plates were sealed and counted in a 1450 Microbeta Jet Liquid Scintillation and Luminescence Counter (Wallac).

Mutant Generation Assays

Spontaneous Resistance

Changes in the susceptibilities of *P. aeruginosa* hypersensitive strain (ATCC 35151) to BT_04E11 were monitored using spontaneous resistance in drug-containing agar (52). *P. aeruginosa* hypersensitive strain was grown at 37 °C in 50 mL of TSB for 4 to 6 h or until the concentration of the culture was $OD_{600} > 1$. The bacteria was harvested by centrifugation (5,000 × g for 10 min) and re-suspended in 1-2 mL of TSB and set to 0.5 McFarland standard. Cell suspension was prepared at 10^{10} CFU/mL and 0.1 mL of the suspension was used to inoculate agar plates containing compound BT_04E11 at 4 × MIC w and incubated at 37 °C. Colonies are picked after 96 hr and sub-streaked on fresh blood agar plate if heterogeneous colonies were present. 16 individual colonies were picked, MICs' were recorded per sample and individual colonies were grown in 3 mL of TSB and genomic DNA was extracted from samples.

Serial Passage

Changes in the susceptibilities of *P. aeruginosa* hypersensitive strain to BT_04E11 were monitored using serial passages in drug-containing broth (53). The first passage was a broth microduliton MIC test. For the subsequent passages, visible cell growth in the well with the highest inhibitor concentration was re-suspended and transferred by dipping the tip of the micropipettmen into the culture and transferring into 1 mL of TSB (1,000 –fold dilution). Freshly inoculated TSB sample was used to inoculate 96-well MIC plate. MIC's for a total of 20 daily passages were recorded. After passage 20, the isolate was cultured on blood agar purity plate before determination of the final MIC. From the purity plate 16 individual colonies were selected, grown in 3 mL of TSB and genomic DNA was extracted.

Characterization of Mutants

Individual colonies from spontaneous mutation and serial passage results were used to inoculate 3 mL TSB cultures. 12-18 h post inoculation, 250 μL of sample was used to make glycerol stocks and the remaining bacterium was harvested by centrifuged (10 min at 5,000 × g). Genomic DNA was isolated using the Genomic DNA Purification Kit (Thermo scientific) and concentration was measured using NanoDrop 1000 spectrophotometer (Version3.6.0, Thermo Scientific). Genes were amplified by PCR (Bio-Rad MJ Mini Thermo Cycler) using a forward primer (5'-ctgagctagcgaaaacctggatgcgctf-3') and a reverse primer (5'-gactaagcttctactatttccttaacgtggcattg-3') (Integrated DNA Technologies, Coralville, IA). PCR products were sequenced (Functional Biosciences, Inc.) alongside 3 primers (R346, R792, F712) that were specific for the PheRS alpha subunit from IDT and alignments were performed using Vector NTI Advance ® 11.5 (Invitrogen). Amino acids sequence from individual mutant strains were compared with the gene encoding *P. aeruginosa* PheRS alpha subunit.

Mutational Modeling of *E. coli* PheRS

Sequence alignments were used to map the mutated amino acids found in *P. aeruginosa* PheRS alpha subunit to corresponding residues in *E. coli* PheRS alpha subunit (PDB 3PCO). PyMOL (Delano Scientific) was used to locate amino acid mutations in the 3D structure of *E. coli* PheRS alpha subunit. The *P. aeruginosa* 3D structure was used to show relationship of mutated amino acids to the active site of the alpha-subunit and to the beta-subunit.

Structural Activity Relationship (SAR) Development

Structural activity relationship (SAR) compounds were identified from the Ambinter chemical compound database. The four leading compounds: BT_04E11, BT_06G06, BT_06D07, and BT_08F11 were the base for identification of 16 SAR compounds (4 per lead compound). These sixteen compounds were acquired and the IC_{50}'s were determined. MIC values were determined against a panel of 10 pathogenic bacteria as described above.

CHAPTER IV

RESULTS

Screening of Synthetic Chemical Compound Library: Timtec

Initial Screening

Using the optimized A/T system, 890 chemical compounds from a synthetic compound library, was screened as described in 'Materials and Methods'. Positive controls contained 2 μL DMSO instead of compounds and were located in row 1 columns A-D and row 12 columns E-H in the 96-well plate. Negative controls contained 2 μL 0.5 M EDTA (pH 8.0) and were located on row 1 columns E-H and row 12 columns A-D in the 96-well plate. The remaining 80 wells; rows 2-11 and columns A-H, contained 2 μL of 3.3 mM chemical compound for a final concentration of 132 μM in the assay. The initial screen was conducted as single point assays and a compound that inhibited over 50% of poly-Phe synthesis was defined as a hit.

The results of the synthetic chemical compound library initial screen were plotted using a 3-D scatter graph (JMP Pro 10.0.2) (Figure 5). From the initial screen of the chemical compound library, 79 compounds were identified to have inhibited 50% of the protein synthesis activity based on the DMSO control.

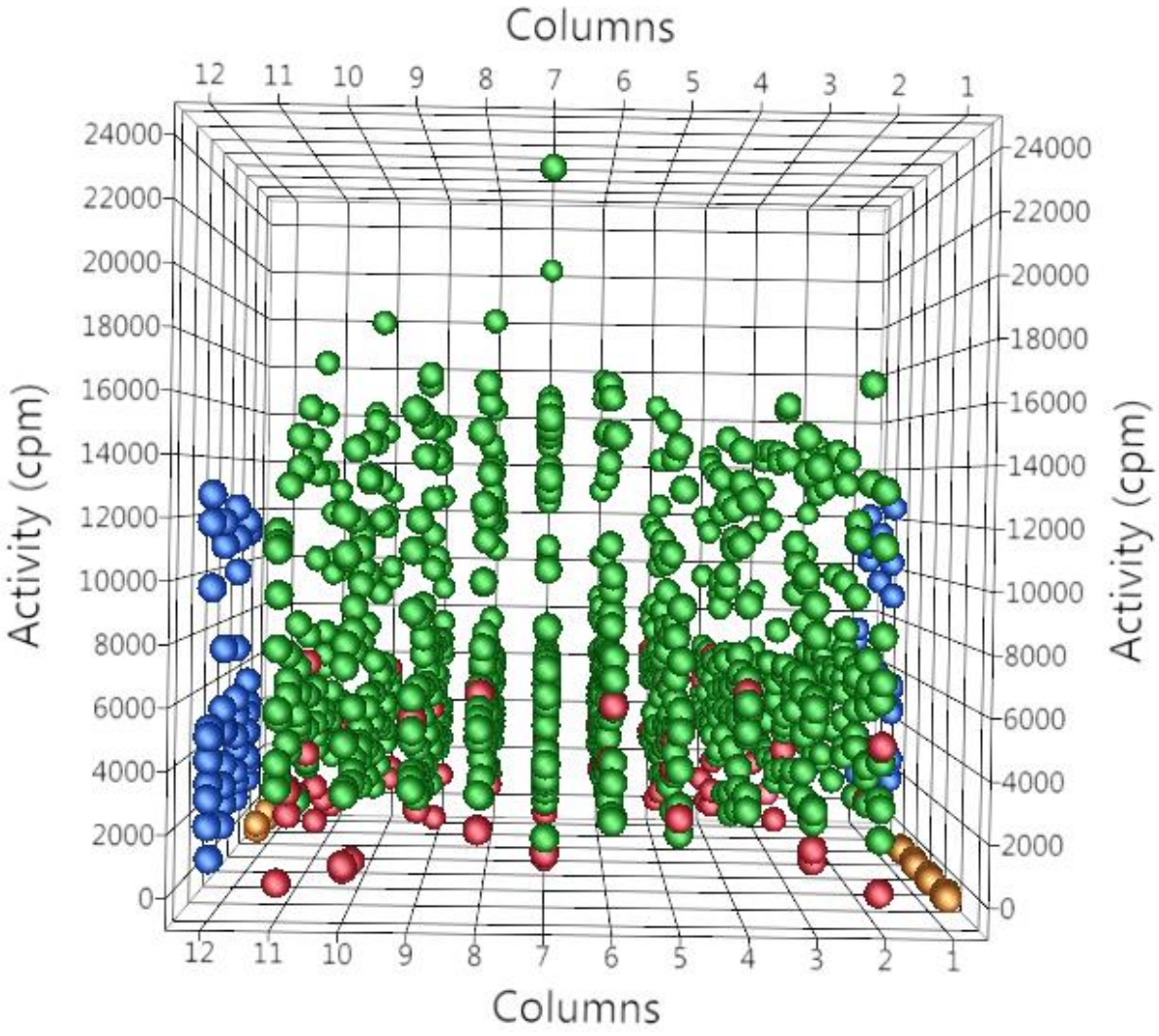


Figure 5. Initial single point screening results of the synthetic chemical compound library. The graphical representation shows DMSO control (blue circles/positive control), EDTA control (orange circles/negative control), primary hits (red circles/activity is 50% below positive control), and other chemical compounds (green circles/ non-hits).

Confirmation of Primary Hits

All primary hits were re-assayed using scintillation proximity assay, as described in initial screening under ‘Material and Methods” with the exception confirmed assay were in duplicate form. Of the 79 initial hits, 36 compounds were confirmed to inhibit greater than 50% of the activity of the system (Figure 6). The 36 compounds were carried forward for additional analysis.

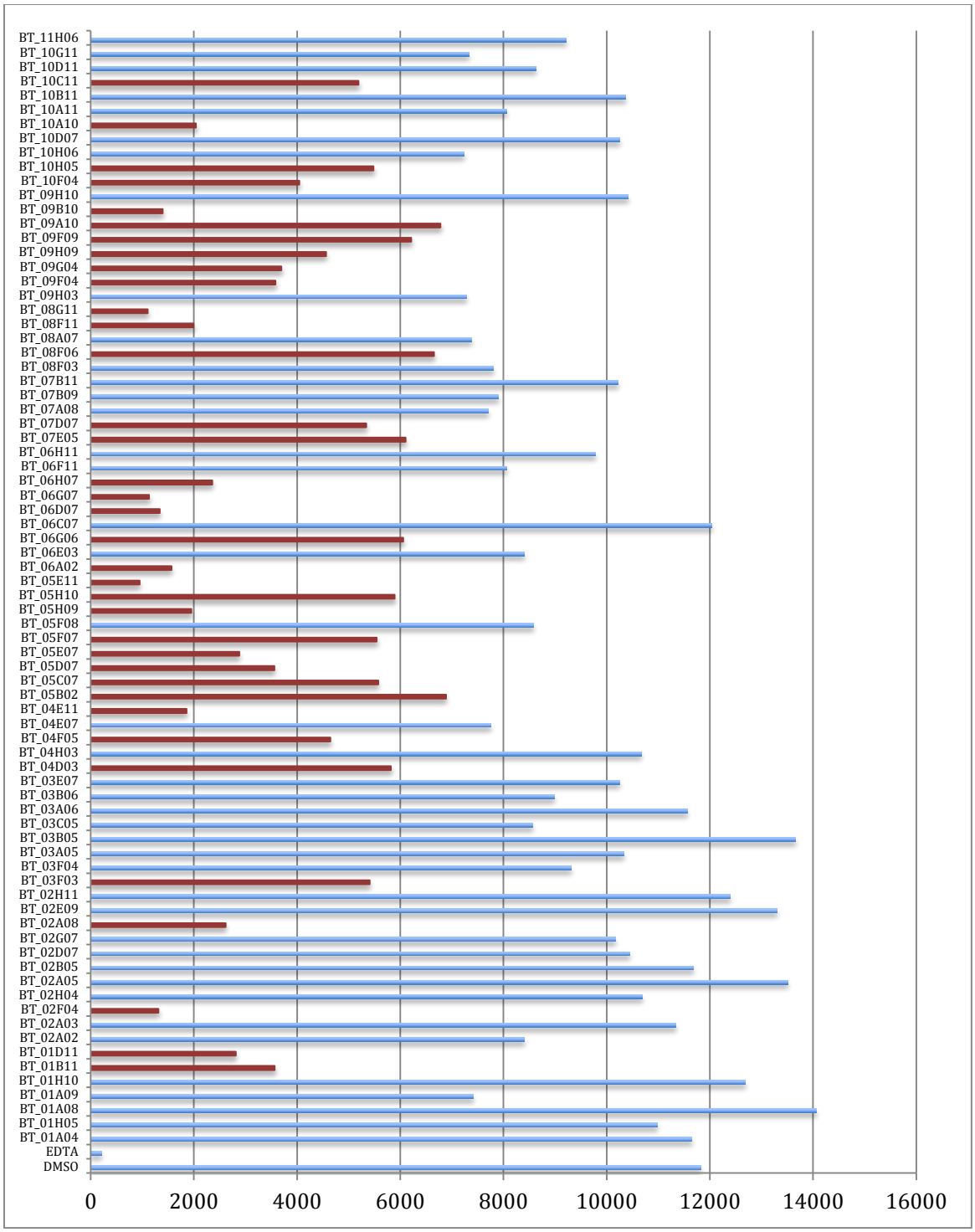
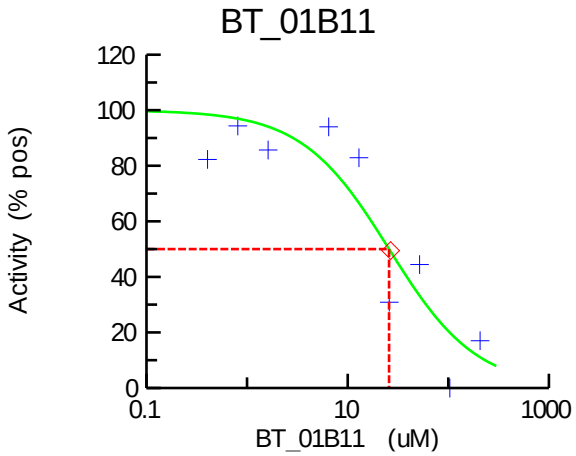


Figure 6. Confirmation of chemical compound library primary hits results. The graph is depicted by blue bars, representing false positives and the red bars, which are the confirmed hits.

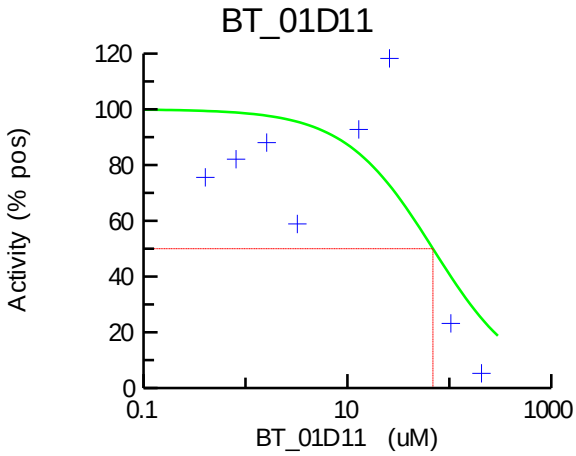
IC_{50} Assay of Chemical Compound Library Confirmed Hits

Assays were performed to determine the concentration of the compound that inhibited 50% of the activity of the system as described under 'Materials and Methods'. Chemical compounds were serially diluted from 5 to 0.01 mM resulting in a final concentration range between 200 to 0.4 μM in the assay plate. The data was analyzed using XL fit (version 5.1;IDBS) as part of Microsoft Excel (Figure 7). IC_{50}'s for the 36 chemical compounds ranged from 7.1 μM to 124.0 μM. The IC_{20} values for the 36 chemical compounds are shown in table 1.

A


BT_01B11
Activity (% pos)
120
100
80
60
40
20
0
0.1
10
1000
BT_01B11 (uM)

B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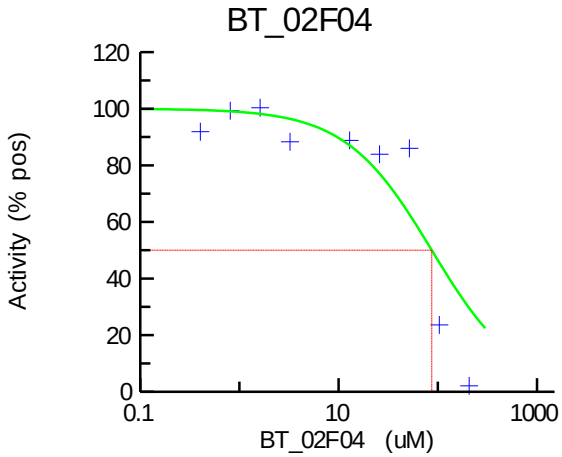
40
20
0
0.1
10
1000
BT_01D11 (uM)

C


BT_02F04
Activity (% pos)
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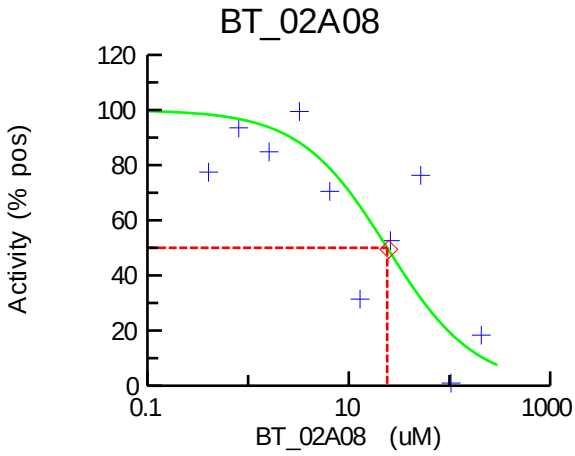
60
40
20
0
0.1
10
1000
BT_02F04 (uM)

D


BT_02A08
Activity (% pos)
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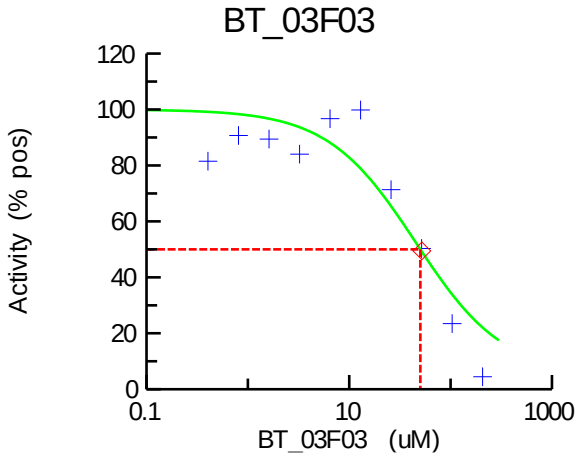
0.1
10
1000
BT_02A08 (uM)

E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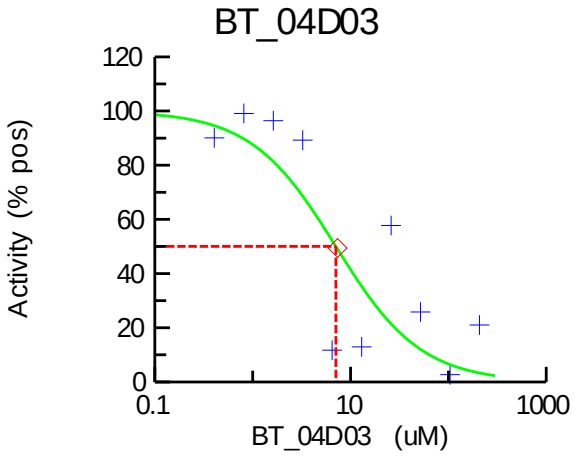
0.1
10
1000
BT_03F03 (uM)

F


BT_04D03
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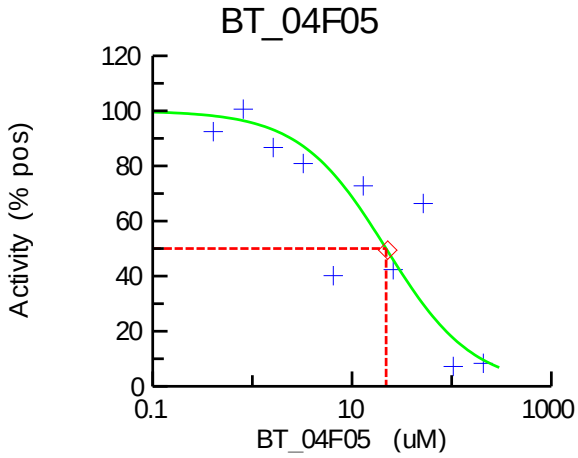
0.1
10
1000
BT_04D03 (uM)

G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Activity (% pos)
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100
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60
40
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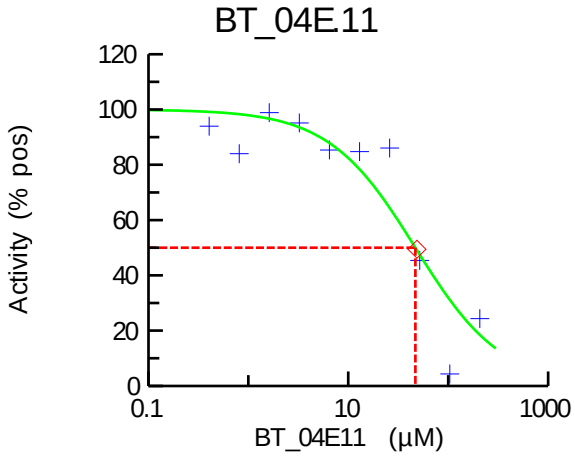
0.1
10
1000
BT_04F05 (uM)

H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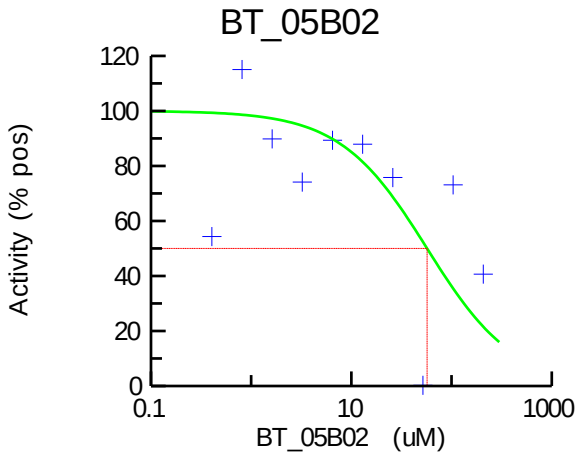
0.1
10
1000
BT_04E11 (µM)

I


BT_05B02
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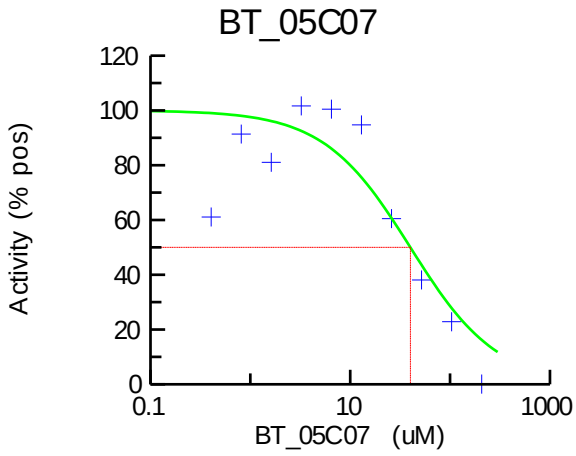
0.1
10
1000
BT_05B02 (uM)

J


BT_05C07
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0.1
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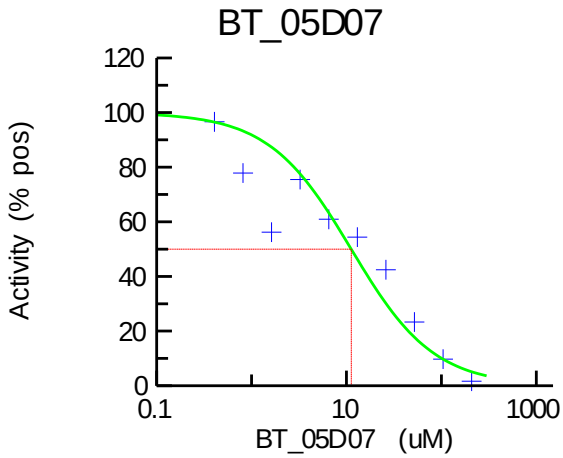
BT_05C07 (uM)

K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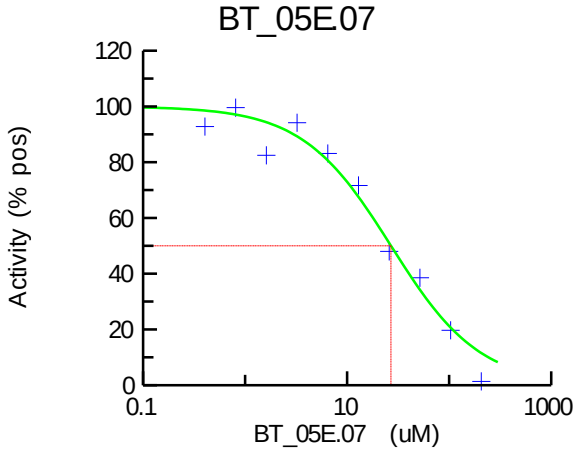
0.1
10
1000
BT_05D07 (uM)

L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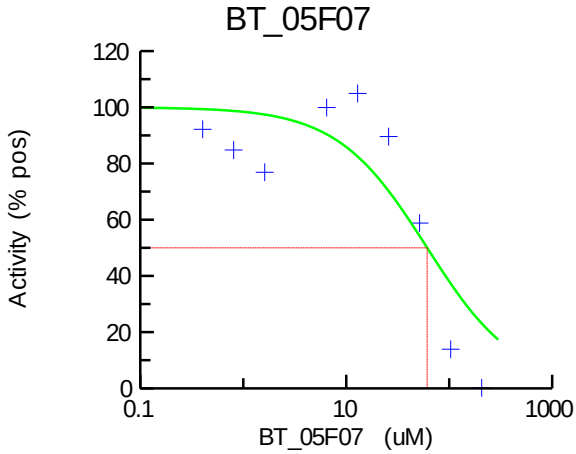
0.1
10
1000
BT_05E.07 (uM)

M


BT_05F07
Activity (% pos)
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0.1
10
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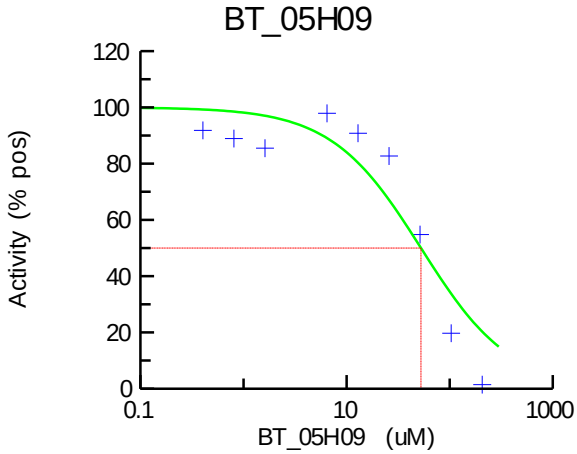
BT_05F07 (uM)

N


BT_05H09
Activity (% pos)
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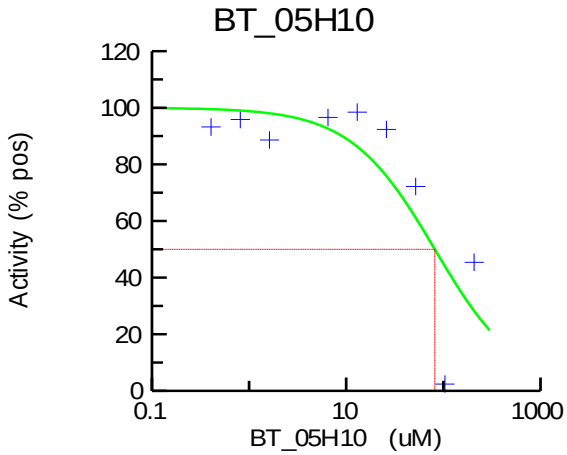
0.1
10
1000
BT_05H09 (uM)

O


BT_05H10
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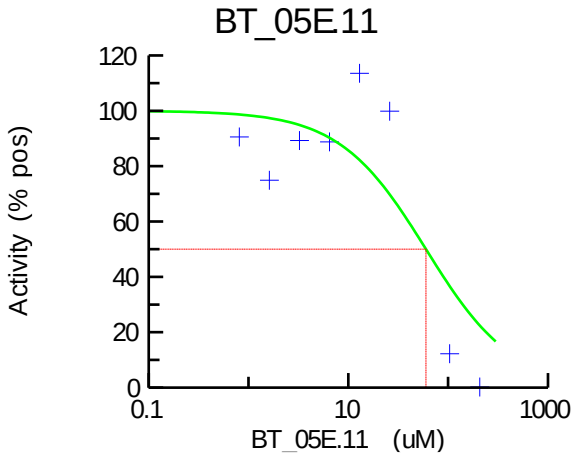
0
0.1
10
1000
BT_05H10 (uM)

P


BT_05E.11
Activity (% pos)
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20
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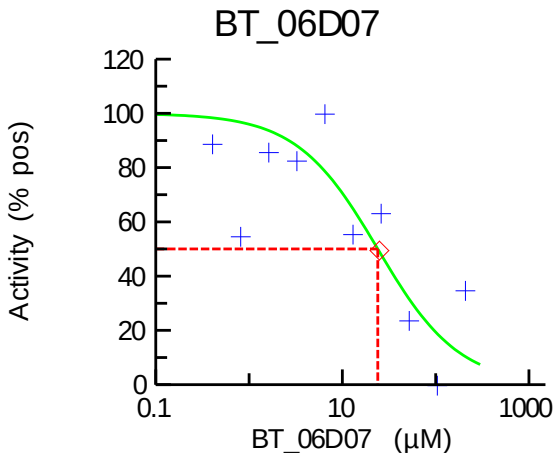
0.1
10
1000
BT_05E.11 (uM)

Q


BT_06D07
Activity (% pos)
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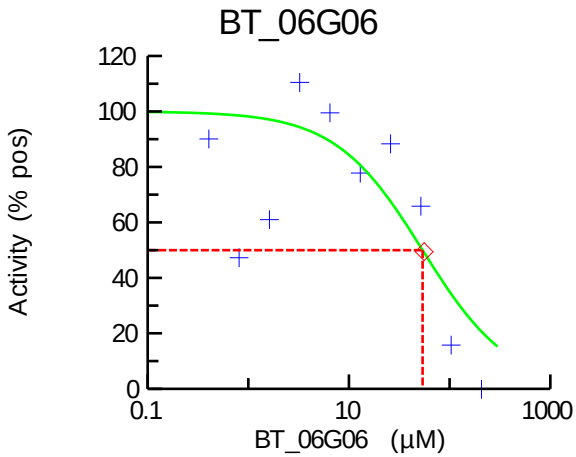
40
20
0
0.1
10
1000
BT_06D07 (µM)

R


BT_06G06
Activity (% pos)
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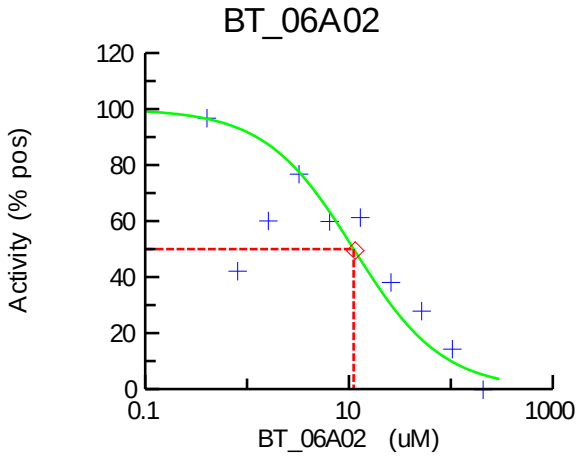
40
20
0
0.1
10
1000
BT_06G06 (µM)

S


BT_06A02
Activity (% pos)
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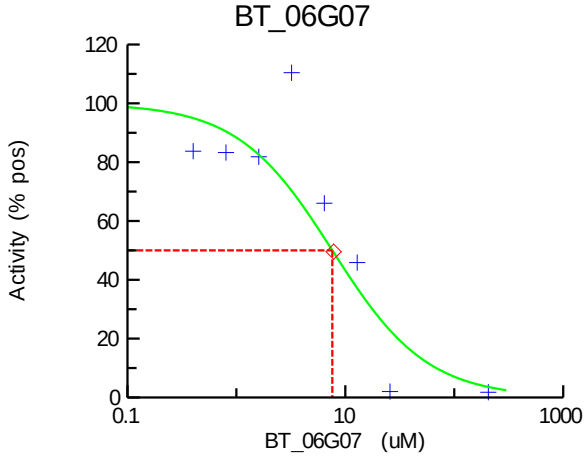
60
40
20
0
0.1
10
1000
BT_06A02 (uM)

T


BT_06G07
Activity (% pos)
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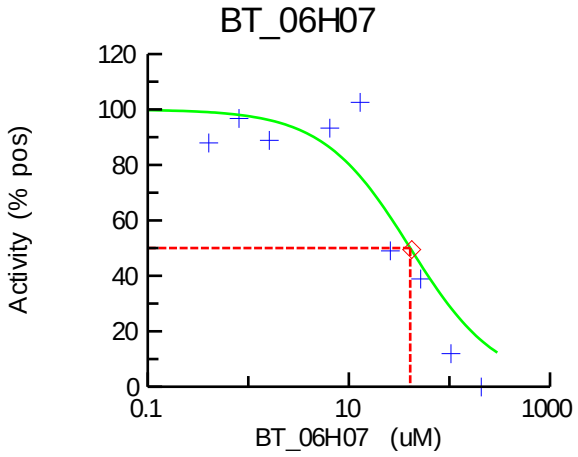
20
0
0.1
10
1000
BT_06G07 (uM)

U


BT_06H07
Activity (% pos)
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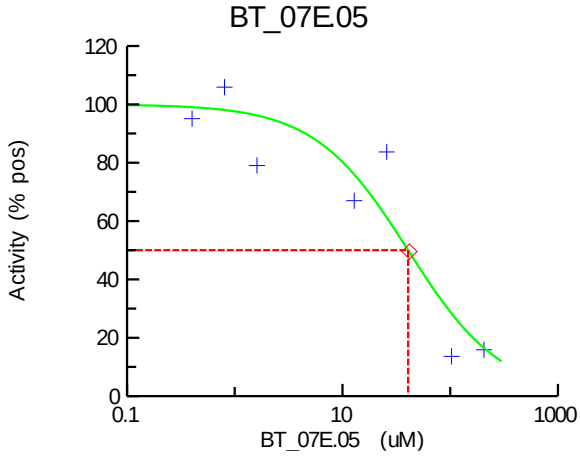
100
80
60
40
20
0
0.1
10
1000
BT_06H07 (uM)

V


BT_07E.05
Activity (% pos)
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40
20
0
0.1
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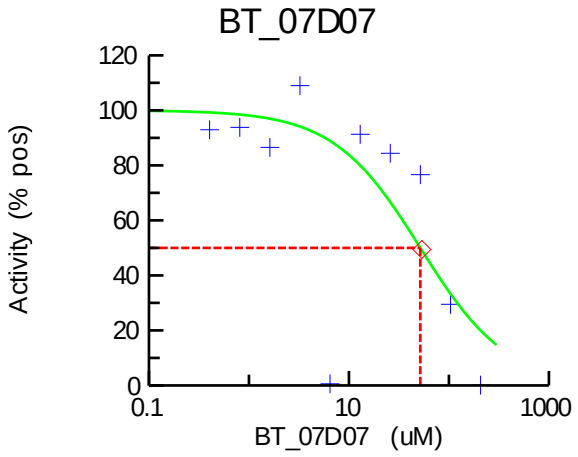
1000
BT_07E.05 (uM)

W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Activity (% pos)
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20
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0.1
10
1000
BT_07D07 (uM)

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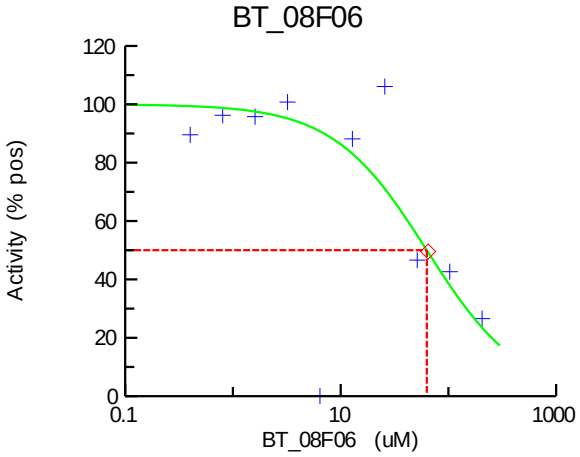
BT_08F06
Activity (% pos)
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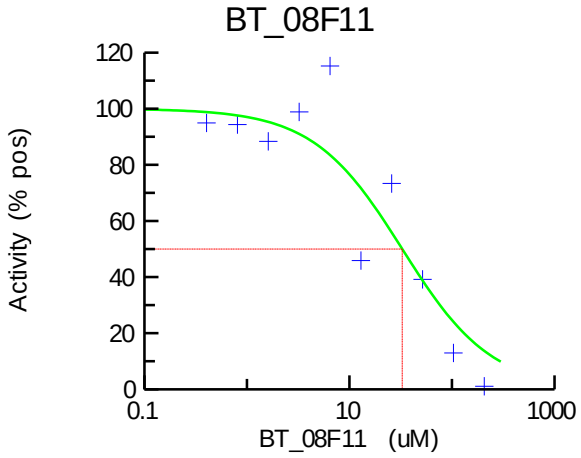
100
80
60
40
20
0
0.1
10
1000
BT_08F06 (uM)

Y


BT_08F11
Activity (% pos)
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0.1
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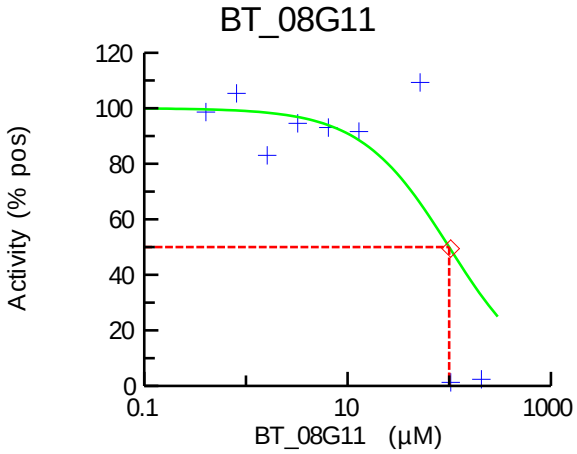
1000
BT_08F11 (uM)

Z


BT_08G11
Activity (% pos)
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0
0.1
10
1000
BT_08G11 (µM)

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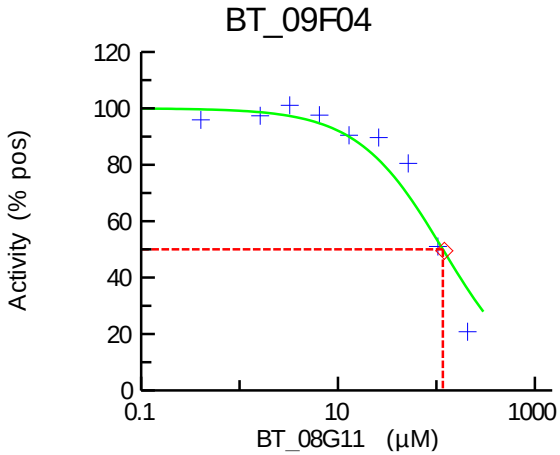
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1000
BT_08G11 (µM)

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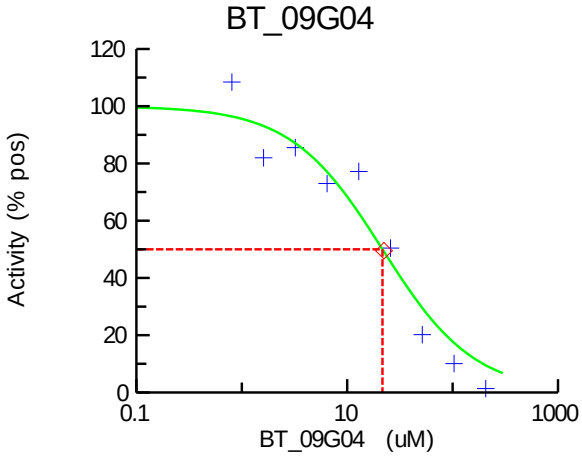
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0.1
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BT_09G04 (uM)

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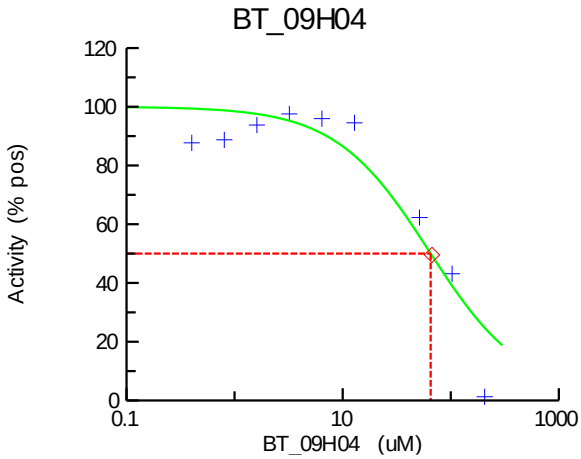
BT_09H04
Activity (% pos)
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20
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0.1
10
1000
BT_09H04 (uM)

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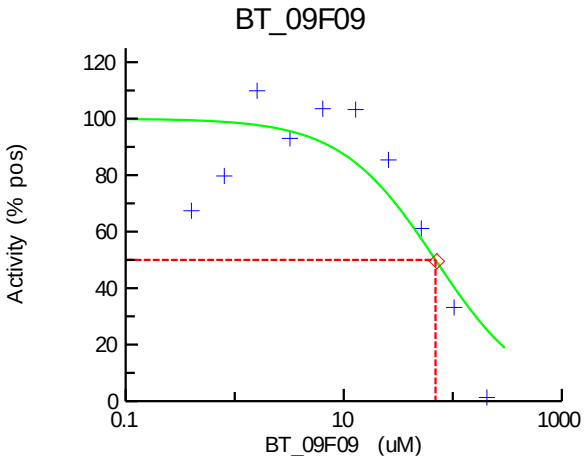
BT_09F09
Activity (% pos)
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40
20
0
0.1
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1000
BT_09F09 (uM)

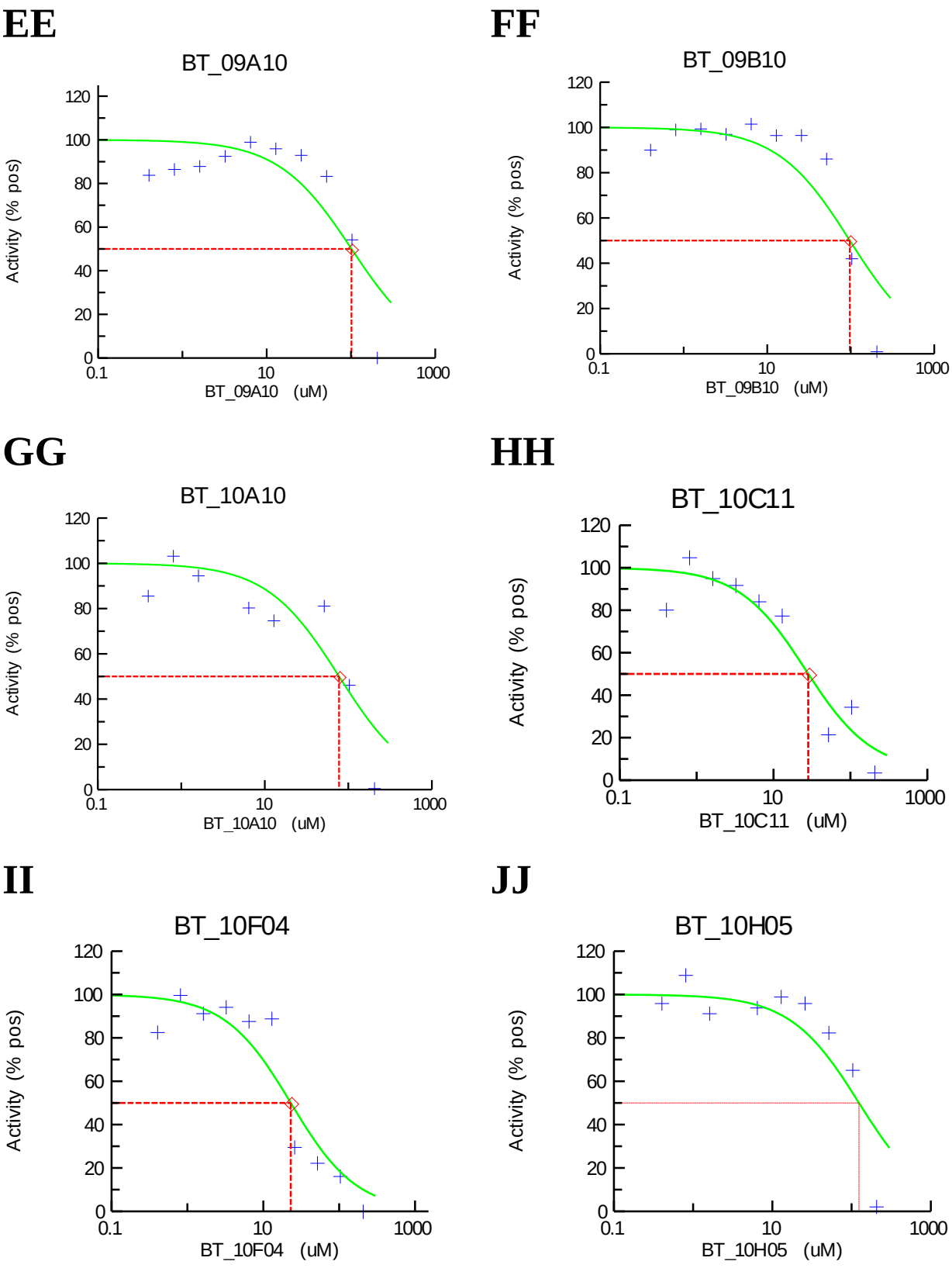


Figure 7. Determination of IC_{50} of the 36 chemical compounds confirmed from the initial screen.

Chemical Compounds	IC_{50} Value (µM)
BT_01B11	**25.8**
BT_01D11	**69.1**
BT_02F04	**86.9**
BT_02A08	**24.1**
BT_03F03	**50.3**
BT_04D03	**7.10**
BT_04F05	**22.0**
BT_04E.11	**47.0**
BT_05B02	**57.0**
BT_05C07	**40.0**
BT_05D07	**11.2**
BT_05E07	**26.9**
BT_05F07	**61.2**
BT_05H09	**52.7**
BT_05H10	**81.7**
BT_05E.11	**59.9**
BT_06A02	**11.1**
BT_06G06	**53.9**
BT_06D07	**24.0**
BT_06G07	**7.6**
BT_06H07	**40.8**
BT_07E.05	**40.7**
BT_07D07	**51.8**
BT_08F06	**63.0**
BT_08F11	**32.8**
BT_08G11	**99.7**
BT_09F04	**116.4**
BT_09G04	**21.6**
BT_09H04	**65.3**
BT_09F09	**69.5**
BT_09A10	**101.6**
BT_09B10	**97.5**
BT_10F04	**23.0**
BT_10H05	**124.0**
BT_10A10	**77.4**
BT_10C11	**28.3**

Table 1. IC_{50} values of chemical compound hits from Timtec library.

Target determination of hit compounds

Prior to determining hit compounds specific accessory protein inhibition, 13 compounds were noticed to have structure similarity (Figure 8). Upon gathering compound structure of hit compounds, some compounds were noticed to have a similar structure formulation; the base of the compound structure was conserved amongst some compounds while branches of functional groups were changed from one structure to the next. Five groups were created of thirteen structure similarity compounds and only one compound per group was chosen based on having lowest IC_{50} value within its structural similarity group, thus leaving eight compounds out for further testing.

In the A/T system, there are four accessory proteins, disregarding the ribosome, that are required for the synthesis of the poly(Phe) peptide: PheRS, EF-Tu, EF-Ts, and EF-G. Specialty assays (PheRS aminoacylation assay, EF-Tu/EF-Ts GDP exchange assay and EF-G GTPase assay) were used to determine if the function (or functions) of any of the accessory proteins were inhibited.

28 chemical compounds were first tested for inhibitory effects on the activity of PheRS as described under 'Materials and Methods'. Results indicated that 11 compounds inhibit the activity of PheRS but only 3 compounds, BT_01D11, BT_04E11, BT_06G06, were specific for inhibition of PheRS (Figure 9).

The same 28 compounds were treated to determine inhibitory effects against EF-Tu (Figure 10). Five compounds were observed to inhibit the activity of EF-Tu using the GDP-exchange assay. Only one compound, BT_05H10, was observed to have specifically inhibited the activity of EF-Tu.

Next, we assayed the effect the 28 test compounds have on the ribosome dependent GTPase activity of EF-G. The amount of GTPase activity was monitored by the measurement of the amount of P_i liberated using a colorimetric GTPase assay kit (Novus Biologicals) as described under 'Materials and Methods'. Six compounds were observed to inhibit the activity of EF-G in the GTPase assay (Figure 11) but none were specific for EF-G.

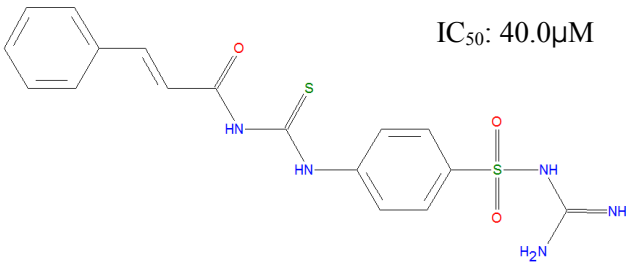
Next compounds that were specific inhibitors of PheRS, were used to test for inhibitory effects on *P. aeruginosa* aa-RS, LeuRS. Procedure was followed as described under 'Materials and Methods'. Results showed three of the six compounds that inhibited the activity of PheRS also inhibited the function of LeuRS function (Figure 12).

The remaining 16 compounds did not inhibit activity of accessory protein represented in the coupled aminoacylation/translation (A/T) screening system but were confirmed to inhibit poly-Phe synthesis in the biochemical assays, therefore leading to the conclusion that the likely mechanism of action of these compounds was direct inhibition of the ribosome itself.

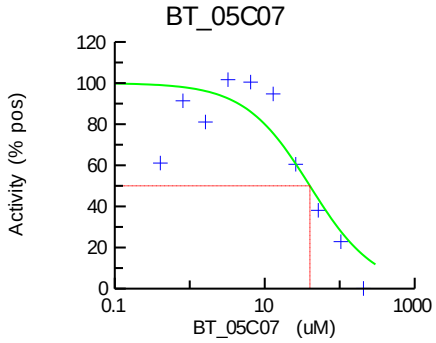
A total of 8 compounds were shown to inhibit more than one accessory protein and these were therefore not analyzed further. IC_{50} value and molecular target of confirmed compound hits are listed in table 3.

Group 1:

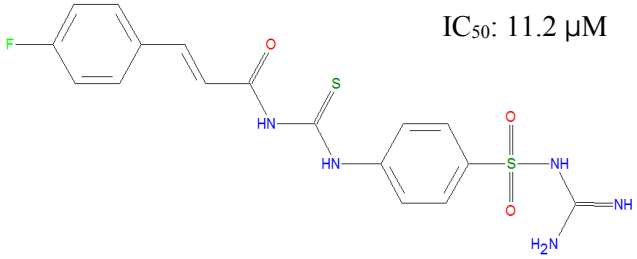
BT_05C07



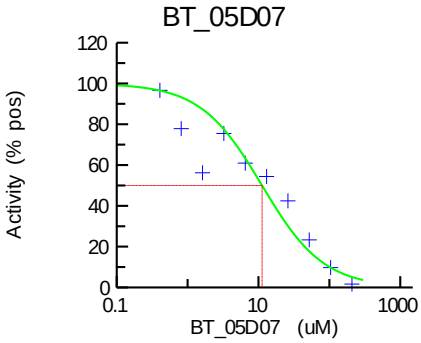
IC_{50}: 40.0μM



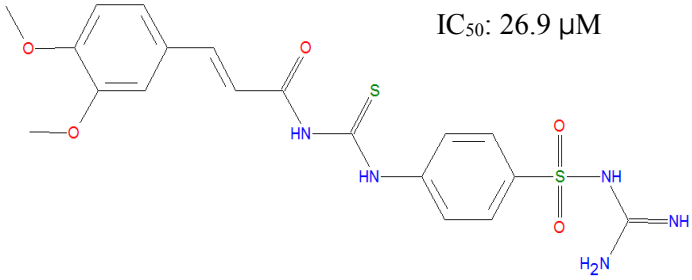
BT_05D07



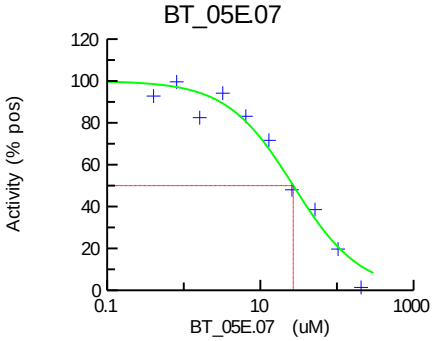
IC_{50}: 11.2 μM



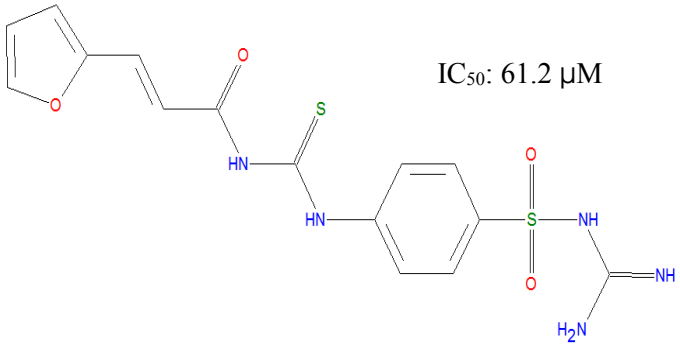
BT_05E07



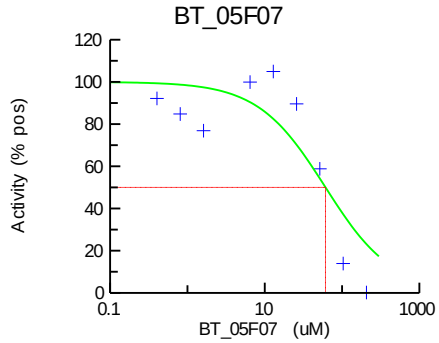
IC_{50}: 26.9 μM



BT_05F07



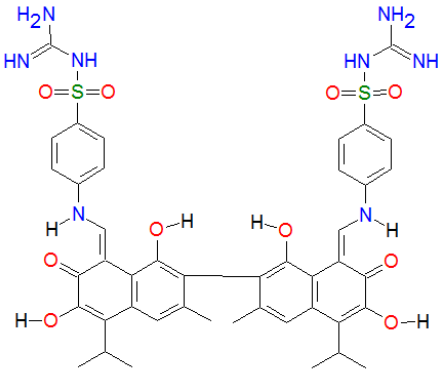
IC_{50}: 61.2 μM

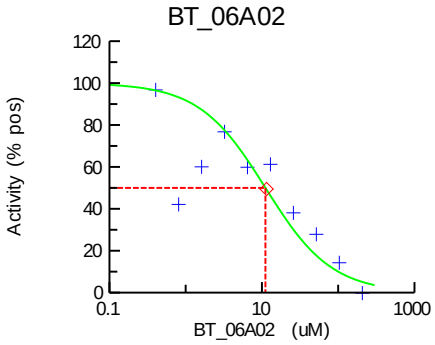


Group 2:

BT 06A02

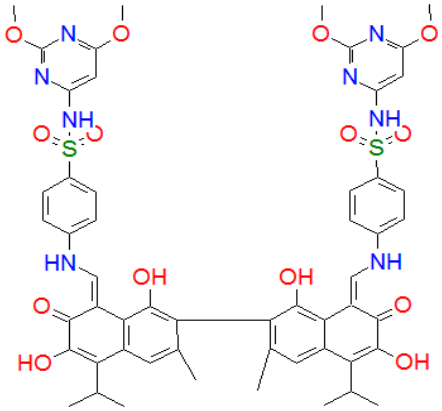
IC_{50}: 11.1 μM

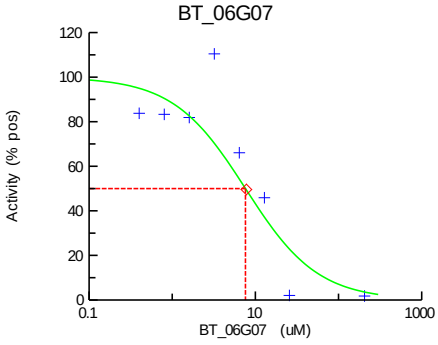




BT_06G07

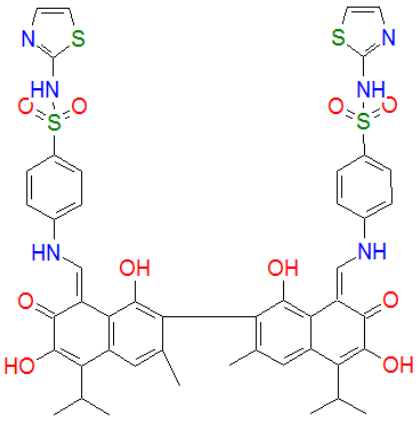
IC_{50}: 7.6 μM

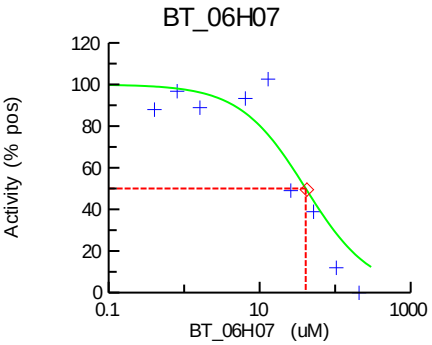




BT_06H07

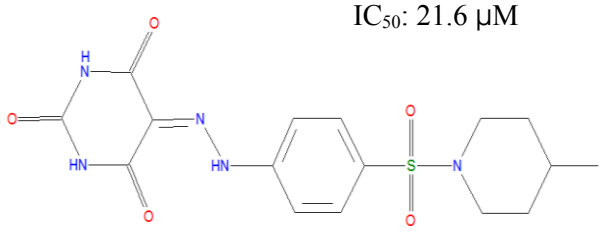
IC_{50}: 40.8 μM



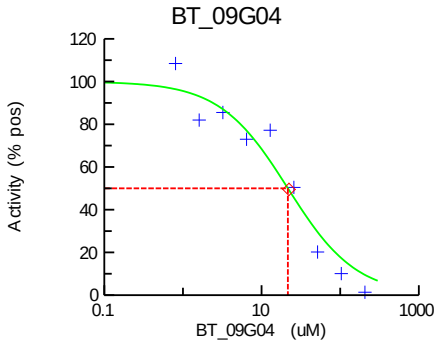


Group 3:

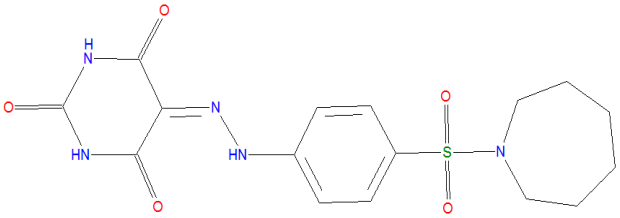
BT_09G04



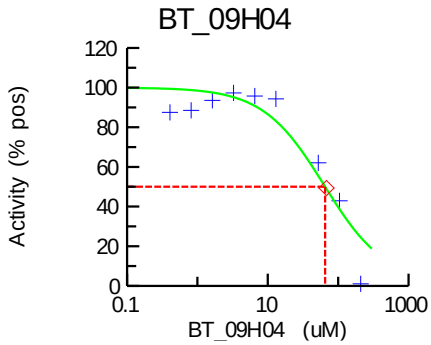
IC_{50}: 21.6 µM



BT_09H04



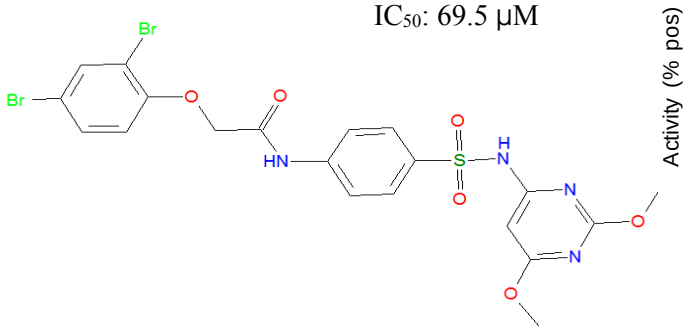
IC_{50}: 65.3 µM

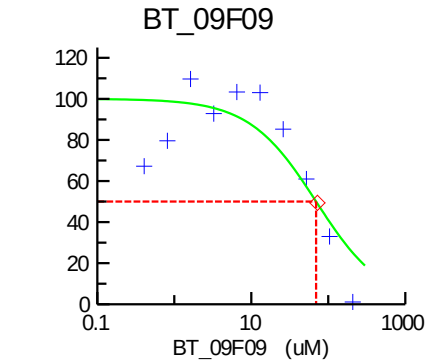


Group 4:

BT_09F09

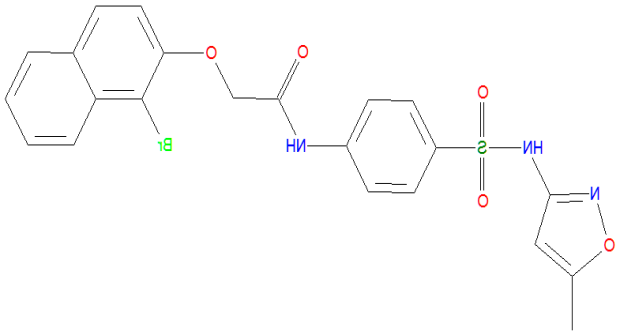
IC_{50}: 69.5 µM

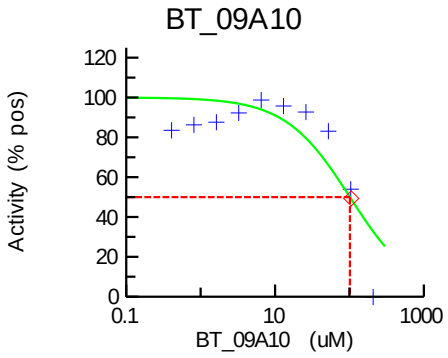




BT_09A10

IC_{50}: 101.6 µM





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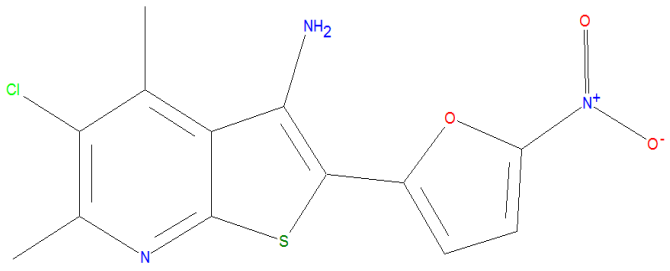
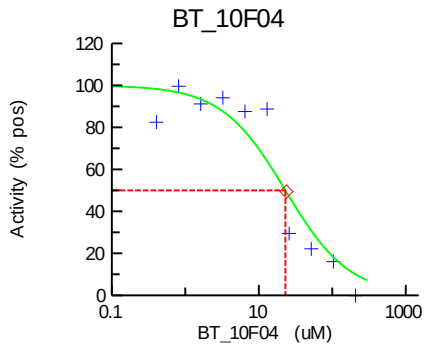
Group 5:

BT_10F04

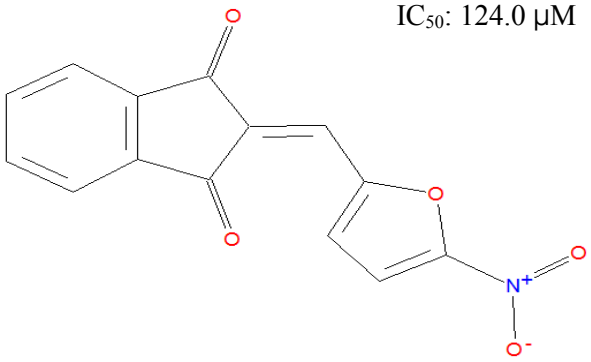
IC_{50}: 23.0 µM





BT_10H05

IC_{50}: 124.0 µM



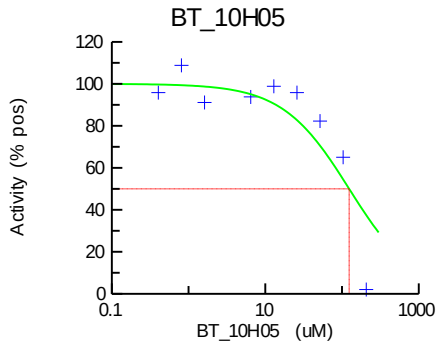


Figure 8. Five groups were developed based on structure similarity. Of these 5 groups, one compound per group was chosen based on lowest IC_{50} value. Theses compounds are outlined by a red box.

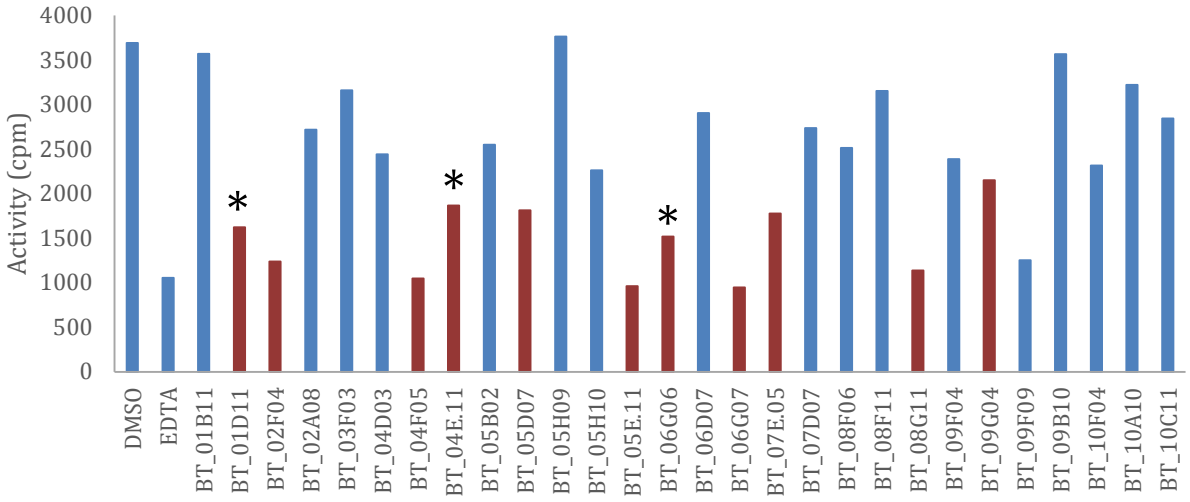


Figure 9. Results of specificity testing of 28 hit compounds against the activity of PheRS using aminoacylation assay. Positive (DMSO) and negative (EDTA) controls are shown. Blue bars are confirmed hit compounds that don't inhibit PheRS, red bars are compounds that inhibit PheRS, and asterisks represent the compounds that are specific for PheRS.

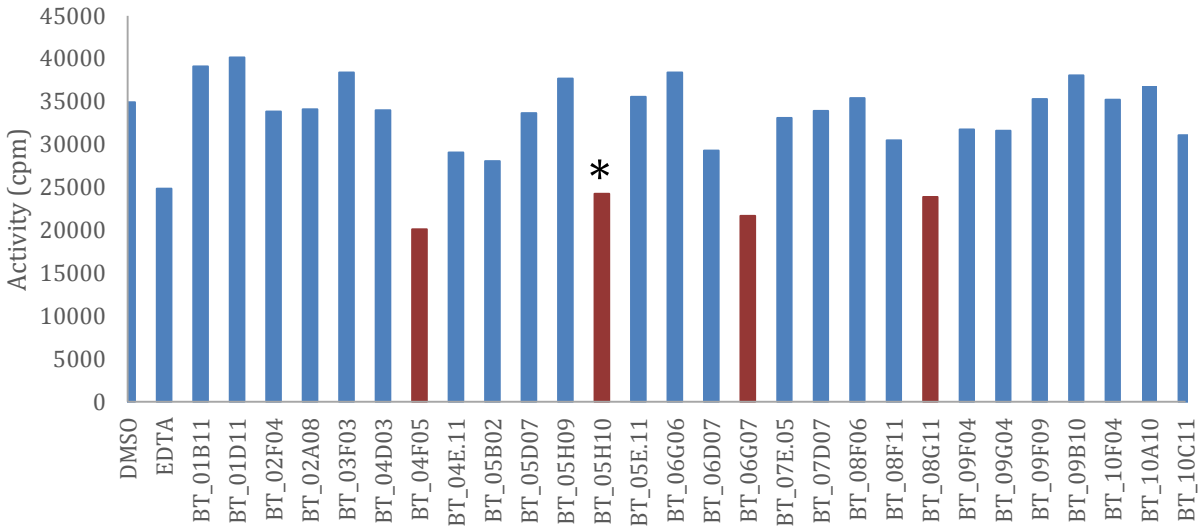


Figure 10. Results of specificity testing against EF-Tu using the GDP-exchange assay. Positive (DMSO) and Negative (EDTA) controls are shown. Blue bars are all the confirmed hit compounds that don't inhibit EF-Tu, red bars are compounds that inhibit the activity of EF-Tu, and asterisks represent the compounds that are specific for EF-Tu.

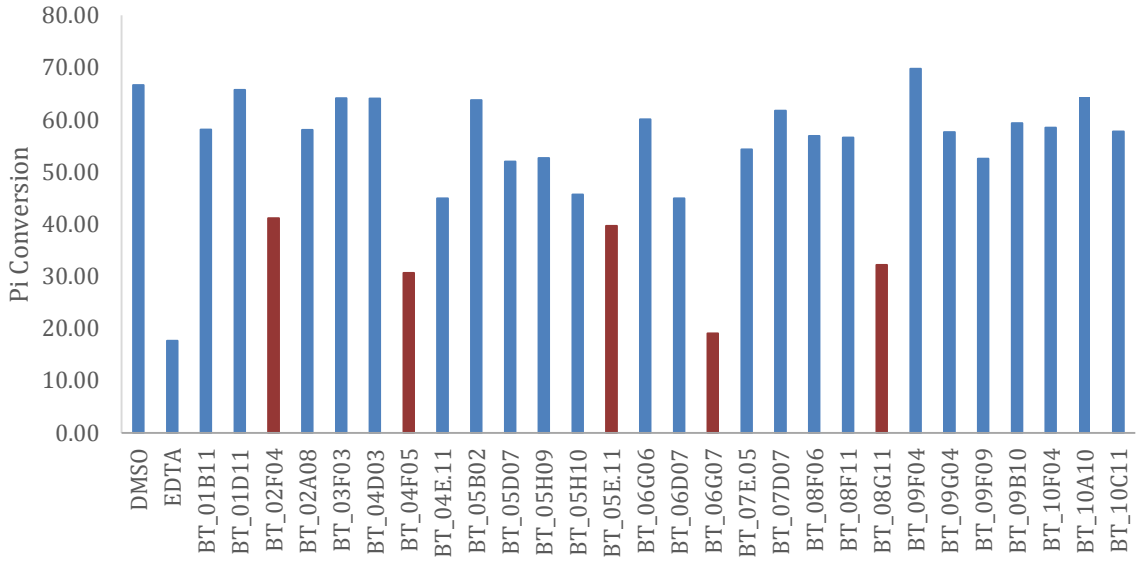


Figure 11. Results of specificity testing against the activity of EF-G. Positive (DMSO) and Negative (EDTA) controls are shown. Blue bars represent confirmed compound hits that did not inhibit EF-G; red bars are compounds that inhibit EF-G.

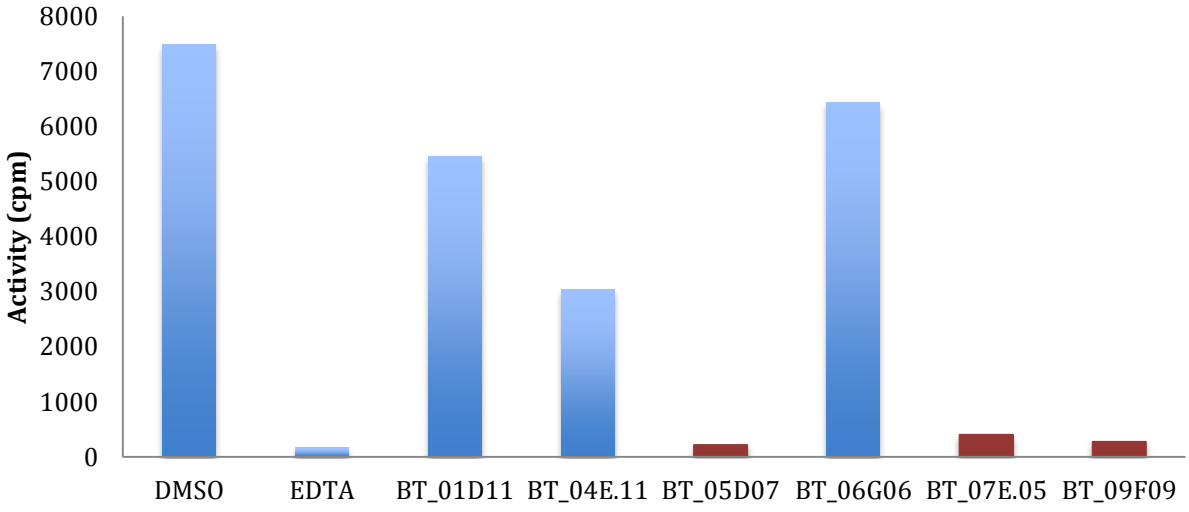


Figure 12. Results of 6 chemical compounds that specifically inhibited PheRS, are tested against the activity of LeuRS using aminoacylation assays. Positive (DMSO) and Negative (EDTA) controls are shown. Blue bars are all the confirmed compound hits that inhibit PheRS but not LeuRS, red bars are compounds that inhibit LeuRS activity.

Chemical Compounds	PheRS	LueRS	EF-Tu	EF-G	Ribosome	IC_{50} Value (μM)
BT_01B11					X	**25.8**
BT_01D11	X					**69.1**
BT_02F04	**X**			**X**		**86.9**
BT_02A08					X	**24.1**
BT_03F03					X	**50.3**
BT_04D03					X	**7.1**
BT_04F05	**X**		**X**	**X**		**22.0**
BT_04E.11	X					**47.0**
BT_05B02					X	**57.0**
BT_05D07	**X**	**X**				**11.2**
BT_05H09					X	**52.7**
BT_05H10			X			**81.7**
BT_05E.11	**X**			**X**		**59.9**
BT_06G06	X					**53.9**
BT_06D07					X	**24.0**
BT_06G07	**X**		**X**	**X**		**17.1**
BT_07E.05	**X**	**X**				**40.7**
BT_07D07					X	**51.8**
BT_08F06					X	**63.0**
BT_08F11					X	**32.8**
BT_08G11	**X**		**X**	**X**		**99.7**
BT_09F04					X	**116.4**
BT_09G04					X	**80.7**
BT_09F09	**X**	**X**				**69.5**
BT_09B10					X	**97.5**
BT_10F04					X	**23.0**
BT_10A10					X	**77.4**
BT_10C11					X	**28.3**

Table 2. Targets and IC_{50} values of confirmed compounds inhibiting A/T system. Black X/black compound represent compounds inhibiting more than one enzymatic component. Red X/red compound represent compounds that are inhibiting specific accessory protein.

MIC Testing of Hit Chemical Compounds

The 20 chemical compounds observed to have inhibited a compound of the A/T system were tested in broth microdilution assays, to determine minimum inhibitory concentrations (MIC). Test compounds were tested against a panel of 10 pathogenic bacteria. Despite similarities in biochemical activity of the compounds, the ability to inhibit bacterial growth varied widely (Table 3). Results reflect high MIC value (≥64 μg/mL) for all compounds tested against *P. aeruginosa PA200 Efflux* (efflux pump mutant), wild type *E. coli*, and *M. cat*. Majority of compounds inhibited *E. coli TolC Efflux*, *P. aeruginosa*, and *P. aeruginosa* Hypersensitive at MIC value (32-1 μg/mL). Overall compounds BT_01D11, BT_02A08, BT_05H10, BT_07D07, BT_08F06, BT_09F04, BT_09G04, and BT_10C11 had high MIC values across the panel of the 10 different pathogenic bacteria. Compounds BT_04E11, BT_06G06, BT_06D07, and BT_08F11 inhibited a wide range of pathogenic bacteria at relatively low MIC's.

MIC Value (µg/mL)										
Chemical Compound	**M. cat**	**H. flu**	**E. coli**	**S. aureus**	**S. pneumo**	**E. faecalis**	**E.coliTolC Efflux**	**P. aeruginosa**	**P.aeruginosa Hypersensitive**	**P.aeruginosa PA200Efflux**
BT_01B11	64	>128	128	64	128	64	4	64	64	64
BT_01D11	128	128	128	128	128	128	128	128	64	128
BT_02A08	128	>128	128	128	128	32	64	64	32	64
BT_03F03	>128	128	128	128	128	64	64	16	>128	64
BT_04D03	128	>128	128	16	128	128	64	64	64	128
BT_04E.11	128	2	128	1	8	128	0.5	2	16	128
BT_05B02	128	>128	128	64	128	64	128	64	32	128
BT_05H09	64	64	128	64	64	128	32	128	16	128
BT_05H10	128	128	128	128	>128	64	128	128	32	128
BT_06G06	32	32	128	32	128	128	2	16	128	128
BT_06D07	64	16	128	16	32	128	2	64	32	128
BT_07D07	128	>128	128	128	128	128	64	64	32	64
BT_08F06	128	>128	128	128	128	64	128	32	32	64
BT_08F11	64	32	64	32	32	16	16	64	128	32
BT_09F04	128	128	128	64	128	128	32	128	32	64
BT_09G04	>128	128	128	32	>128	64	32	128	32	64
BT_09B10	64	>128	128	128	>128	64	1	128	4	128
BT_10F04	64	128	128	64	64	64	1	64	64	64
BT_10A10	128	64	128	32	64	16	8	64	64	128
BT_10C11	128	>128	128	32	128	64	128	64	32	64

Table 3. Minimal inhibitory concentration of 20 chemical compounds against panel of 10 pathogenic bacteria.

Time-Kill Study

Time-kill studies reveal whether a compound is bacteriostatic or bactericidal over a period of 24 hours. Based on the MIC results, compounds were picked based on having the greatest levels of inhibition (low MIC values). Time-kill studies were performed at four times the MIC for the four compounds BT_04E11, BT_06G06, BT_06D07, and BT_08F11. These four compounds were tested against one Gram-negative pathogen, *H. influenza* (Figure 13), and against one Gram-positive pathogen, *S. aureus* (Figure 14).

Compound BT_06D07 was shown to completely inhibit growth of *H. influenza* as well as *S. aureus* at 4, 6 and 24 hours; characterizing this compound as bactericidal against both pathogens. Compound BT_06G06 was shown to be bacteriostatic against both *H. influenza* and *S. aureus*. Although compounds BT_04E11 was bacteriostatic against *H. influenza* results for *S. aureus* at 24 h, compound BT_04E11 becomes bactericidal. Compound BT_08F11 was observed to have bactericidal activity against both *H. influenza* and *S. aureus*; however, as time lapsed, growth of bacteria was observed. Viable bacterial growth at 24 hour may be due to lack of complete inhibition by the compound on bacterial growth in the early phase (0 and 2 hour) thus allowing some re-growth at longer time exposures The results suggest that compound BT_08F11 is bacteriostatic against both *H. influenza* and *S. auerus* but at higher concentrations complete inhibition could be observed.

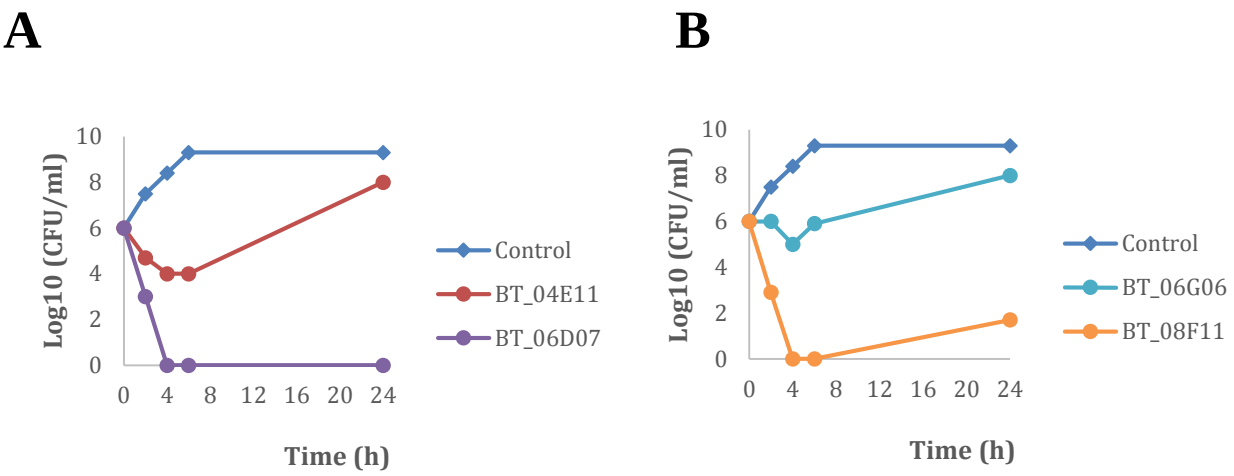


Figure 13. Time-kill kinetics of compound hits against *H. influenza*. Compounds were added to bacterial cultures at four times the MIC. Samples were analyzed by plating and determination of colony forming units (CFU) at 0, 2, 4, 6, and 24 h. Diamonds (♦) represent growth of control culture (absence of inhibitory), filled circles (●) represent cultures containing the test compounds.

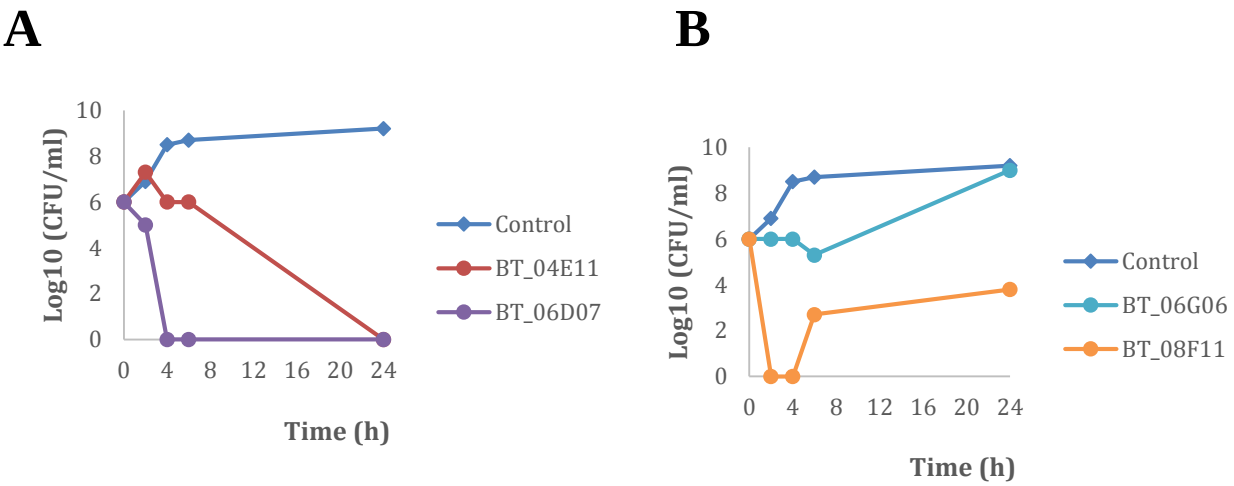


Figure 14. Time-kill kinetics of compound hits against *S. aureus*. Compounds were added to bacterial cultures at four times the MIC. Samples were analyzed by plating and determination of colony forming units (CFU) at 0, 2, 4, 6, and 24 h. Diamonds (♦) represent growth of control culture (absence of inhibitory), filled circles (●) represent cultures containing the test compounds.

Binding Mode Assays

IC_{50}'s for the two compounds that specifically inhibited PheRS (BT_04E11 and BT_06G06) were determined at various concentrations of ATP and Phe in order to determine the mechanism of action of the inhibitors relative to its substrates. Using tRNA aminoacylation assays and varying concentrations of ATP (25, 50, 100, 250, 500 and 1000 μM), the mechanism of action of the two compounds with respect to ATP was determined to be competitive inhibition by compound BT_04E11 (Figure 15A) and noncompetitive by BT_06G06 (Figure 15B) compounds. To determine the mechanism of inhibition with respect to the amino acid, the same assays were used with the exception that ATP was held constant at a saturated concentration of 2.5 mM and IC_{50}'s were determined at varying concentrations of Phe (25, 50, 200, 200 and 300 μM). The mechanism of action of the two compounds with respect to Phe was determined to be noncompetitive by compound BT_04E11 (Figure 16A) and BT_06G06 (Figure 16B).

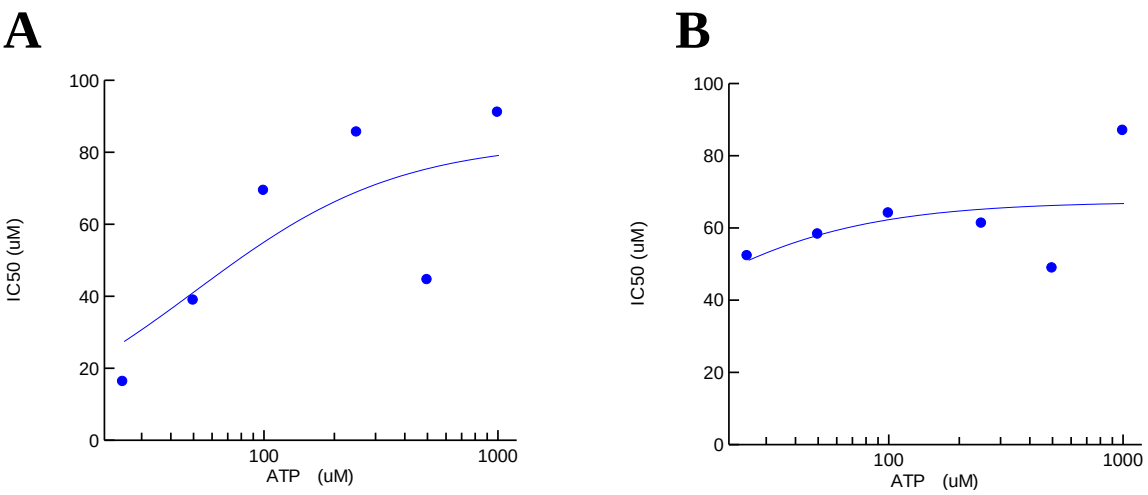


Figure 15. Determination of mechanism of action of the inhibitors relative to ATP. IC_{50}'s for BT_04E11 (A) and BT_06G06 (B) were determined using the aminoacylation assay. The phenylalanine concentration was fixed at 100 μM and IC_{50}'s were determined at six different ATP concentrations ranging from 25 to 1000 μM. The data was fit to the Michaelis-menten steady state model using XLfit (IDBS).

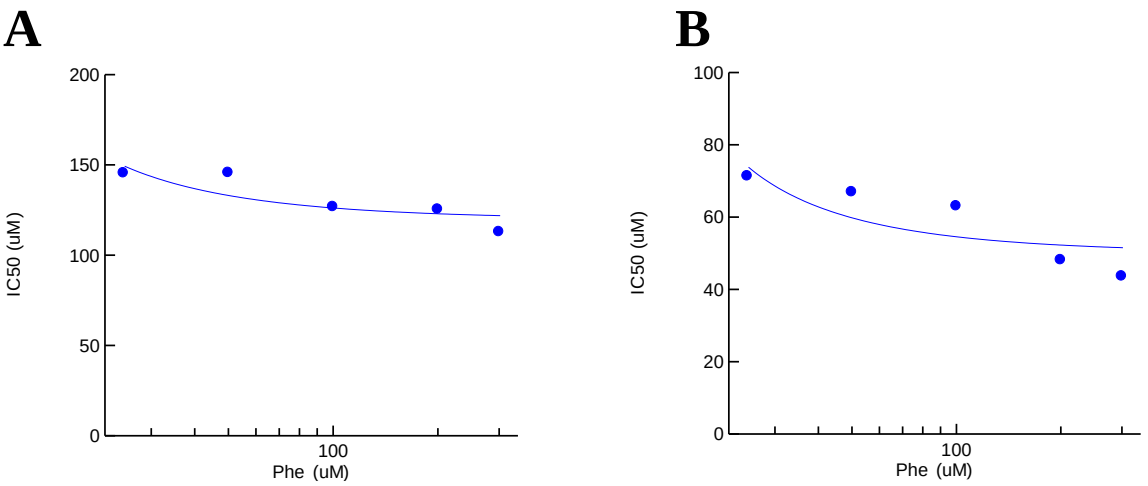


Figure 16. Determination of mechanism of action of the inhibitors relative to Phe. IC_{50}'s for BT_04E11 (A) and BT_06G06 (B) were determined using the aminoacylation assays. The ATP concentration was fixed at 2.5 mM and IC_{50}'s were determined at five different Phe concentrations ranging from 25 to 300 μM. The data was fit to the Michaelis-menten steady state model using XLfit (IDBS).

Eukaryotic Protein Synthesis Assay with Chemical Compound Hits

An ideal antibacterial compound would show potent inhibition against the bacterial target but little or no inhibition in a eukaryotic system.

Human Mitochondrial PheRS Assays. Compound BT_04E11 and BT_06G06 inhibited specifically the accessory protein PheRS in the bacterial protein synthesis system. Since PheRS from human mitochondrial is a homolog of bacterial PheRS and has its own protein synthesis system, it is important to understand possible effects the compounds may have. Compounds BT_04E11 and BT_06G06 were tested against human mitochondrial PheRS (hmPheRS) as described under 'Materials and Methods'. No adverse effect was observed when hmPheRS was screened against the inhibitory compounds up to 200 μM (Figure 17).

Wheat Germ Extract Assays. Compound BT_04E11, BT_06G06, BT_06D07, and BT_08F11 were tested against wheat germ extracts to determine whether they inhibited poly(Phe) synthesis in eukaryotic systems. Results showed that none of the test compounds inhibited protein synthesis in the wheat germ assay at concentrations up to 200 μM (Figure 18).

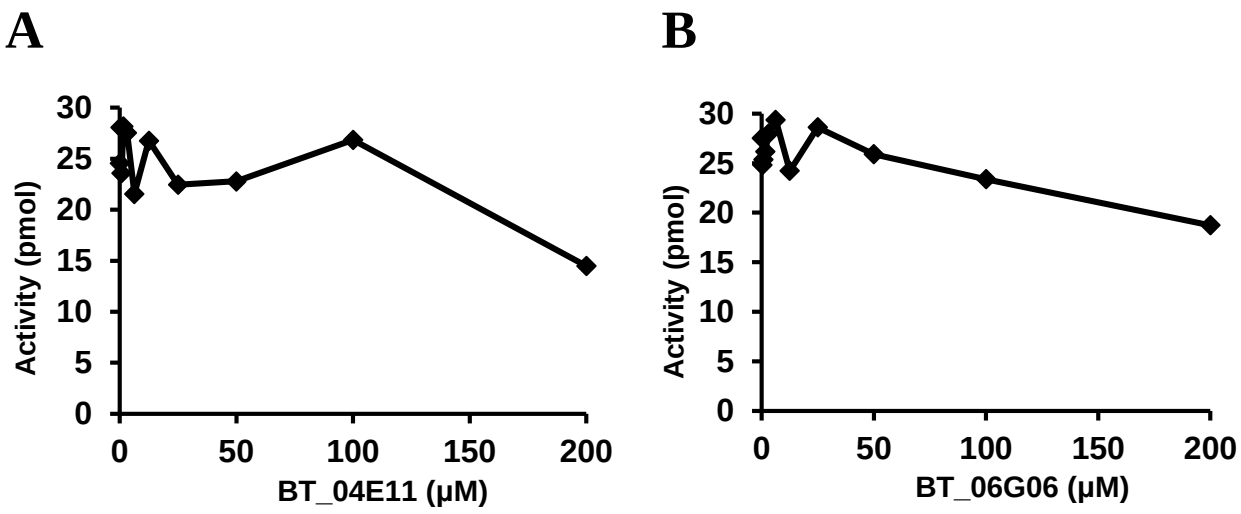


Figure 17. Human mitochondrial PheRS assay of chemical compounds (A) BT_04E11 and (B) BT_06G06.

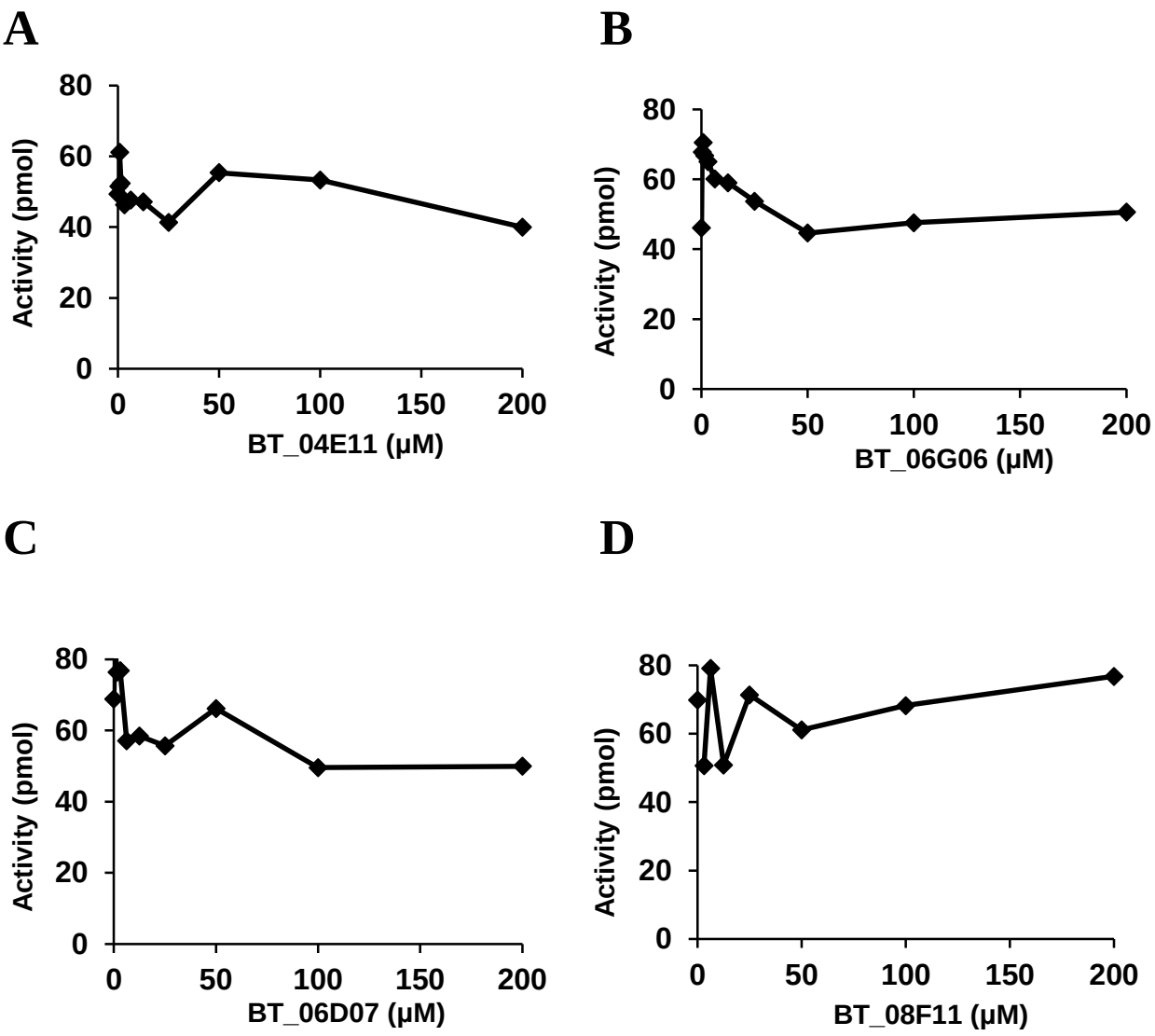


Figure 18. Wheat germ cell extract assays of chemical compound inhbitors. (A) BT_04E11; (B) BT_06G06; (C) BT_06D07; (D) BT_08F11.

In-vitro Cytotoxicity Test

The four lead chemical compounds (BT_04E11, BT_06G06, BT_06D07, and BT_08F11) were tested for possible toxicity in mammalian cells. NIH/3T3 cells were treated with 0.025-1 μg/mL of staurosporine (positive control), 25-200 μg/mL of compounds BT_04E11 and BT_06D07, and 25-125 μg/mL of compounds BT_06G06 and BT_08F11for 24 h under standard tissue culture conditions (Figure 19). Assays were performed in triplicate, results showed the positive control (staurosporine) was toxic to cells at 0.1 μg/mL, to where toxicity was not observed for BT_04E11 at any concentration and toxicity of BT_06D07 was observed at 2000-fold higher concentration than staurosporin. Both compounds BT_06G06 and BT_08F11 were observed to be toxic at approximately 500-fold higher concentration than staurosporin.

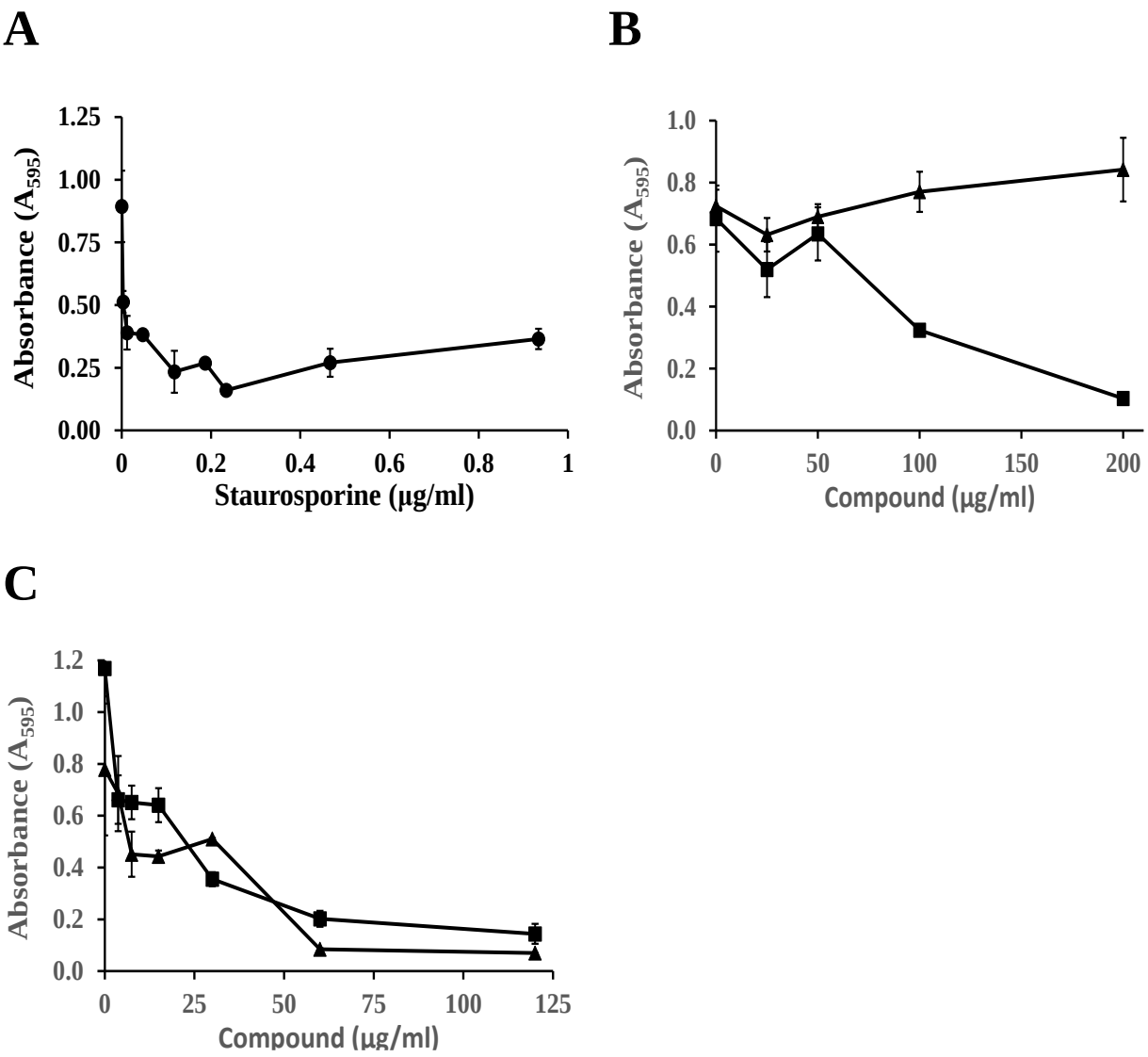


Figure 19. MTT assays to determine toxicity in mammalian cell lines. (A) Staurosporine (positive control); (B) (▲) BT04E11 and (■) BT06D07; (C) (▲) BT06G06 and (■) BT08F11.

Macro-molecular Synthesis (MMS) Assays

Macro-molecular synthesis (MMS) assays were used in the determination of the global mode of action of an inhibitor on the growth of bacteria. MMS assays were carried out on two test compounds (BT_06D07 and BT_08F11) to determine if RNA, DNA, cell wall or protein synthesis was inhibited in bacterial cultures. Assays were evaluated for the compounds effects on macromolecular synthesis in the *Pseudomonas aeruginosa* hypersensitive strain. Compounds BT_04E11 and BT_06G06 demonstrated a dose-dependent inhibition of protein, RNA, DNA and cell wall synthesis, as expected with compounds that elicit the stringent response. Compounds BT_06D07 and BT_08F11 data was compared to the effect tetracycline has on *P. aeruginosa* hypersensitive protein synthesis production. Figure 20A depicts MIC of tetracycline to be 8 μg/mL and MICs for both inhibitors was shown to inhibit at 16 μg/mL. Figure 20B shows the effects of compound BT_06D07 on the production of protein, DNA, RNA and cell wall synthesis. Results show that while all components are inhibited, protein production is inhibited at 8 μg/mL and other components begin to show inhibitory effects prior to protein inhibition. Figure 20C shows the effects of compound BT_08F11 has on the production of the four macro-molecular components. Results suggest that BT_08F11 shows inhibitory effects on protein production at 4 μg/mL and cell wall, RNA and DNA have little to no effect seen in the inhibition of their synthesis. Therefore, compounds BT_06G06 and BT_08F11 are in fact protein synthesis inhibitors.

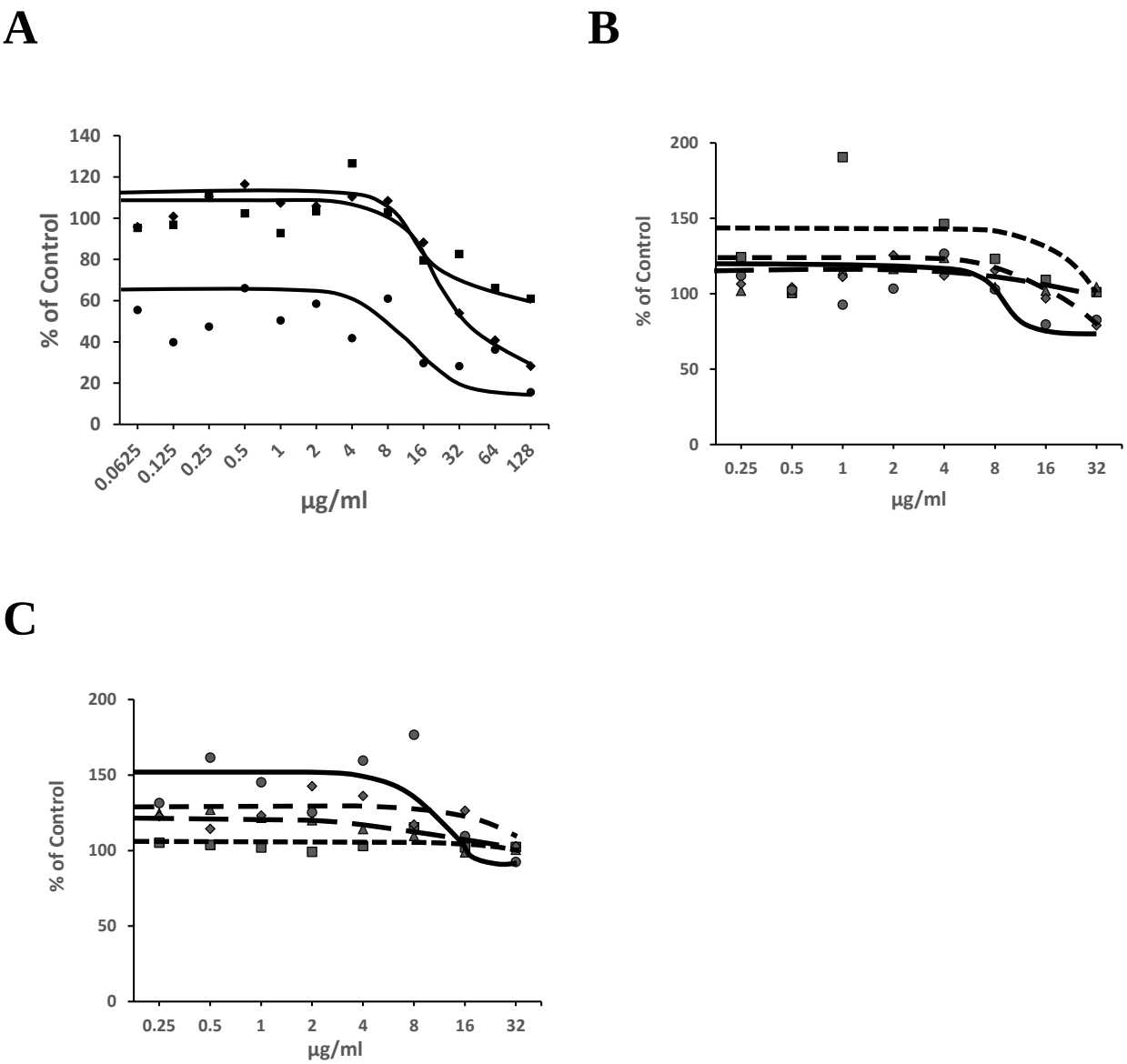


Figure 20. MMS results for compounds BT_06D07 and BT_08F11. (A) (●) Tetracycline (positive control), (▲) BT_06D07 and (■) BT_08F11. (B) BT_06D07 results against (● w/solid line) Protein synthesis, (▲ w/long dash) RNA synthesis, (■ w/small dash) DNA synthesis and (♦ w/medium dash) cell wall synthesis. (C) BT_08F11 results against (● w/solid line) Protein synthesis, (▲ w/long dash) RNA synthesis, (■ w/small dash) DNA synthesis and (♦ w/medium dash) cell wall synthesis.

Mutation Generation Assays

Spontaneous Resistance Assay. *P. aeruginosa* hypersensitive bacteria was evaluated for its propensity to become spontaneously resistant to BT_04E11 when exposed to drug concentrations 4 × MIC. A purity plate was streaked for the separation of individual colonies 96 h post inoculation. 16 individual colonies were chosen from the purity plate and MICs' were determined. The MIC for all bacteria from the colonies ranged from 32 – 128 μg/mL. Using a Genomic DNA Purification Kit (Thermo scientific), genomic DNA was isolated and gene encoding the PheRS alpha subunit from *P. aeruginosa* was amplified by PCR using the Bio-Rad MJ Mini Thermo Cycler. PCR products were sequenced (Functional Biosciences, Inc.) using the 3 primers stated in 'Materials and Methods.' Vector NTI Advance ® 11.5 (Invitrogen) was used for the nucleotide sequence alignments of the mutant forms of the PheRS alpha-subunit with Native form of PheRS alpha subunit (Figures 21). Yellow highlight shows consensus sequence, blue highlight shows variance of nucleotides and within the variance the white highlight depicts the specific nucleotide that varies from individual strains. Strain "10S" was the only isolate to have developed spontaneous resistance amongst the sixteen samples from the alignments using primer R792 (Figure 21). The guanine nucleotide #250 of the *P. aerugionosa* PheRS was mutated to an adenine on the 10th isolate from the spontaneous resistant experimentation. This causes the codon sequence of alanine (GCC) to be changed to a threonine (ACC) at position 84 of the protein sequence of the *P. aeruginosa* PheRS alpha subunit (A84T).

Serial Passage Assay. The propensity of *P. aeruginosa* hypersensitive to develop resistance to BT_04E11 was also evaluated using 20 serial passages. Purity plates were streaked post to the 20th serial passage and 16 individual isolates were chosen. Genomic DNA was extracted as described under 'Materials and Methods' and a gene encoding the PheRS alpha-

subunit was amplified using PCR. Products were sequenced using the 3 primers shown in 'Materials and Methods.' Vector NTI Advance ® 11.5 (Invitrogen) was used for the nucleotide sequence alignments of the mutants from the PheRS alpha-subunit with the native form of PheRS alpha subunit (Figures 21). Three final point mutations were identified all using the R792 primer. Figure 21 shows two mutations in strain 2SP. The first mutation is at the guanine nucleotide #144 of the *P. aerugionosa* PheRS was mutated to an adenine. The second mutation was at the guanine nucleotide #148 of the *P. aerugionosa* PheRS mutating to an adenine. The first mutation causes the codon sequence of leucine (CTG) to be changed to leucine with a different codon sequence (CTA) of position 48 at the PheRS protein (L48L). This is a silent mutation and will not affect the function of the protein, there for removed as a possible mutation. The second mutation causes the codon sequence of alanine (GCC) to be changed to a threonine (ACC) at position 50 of the PheRS protein (A50T). Another point mutation was identified in strain 13SP using R792 primer (Figure 21). The guanine nucleotide #299 of the *P. aerugionosa* PheRS was mutated to an adenine. This resulted in the codon sequence of glycine (GGC) to be changed to aspartate (GAC) at position 100 of the alpha subunit (G100D). The last point mutation shown on figure 21 is in strain 11SP, where a cytosine nucleotide #337 of the *P. aerugionosa* PheRS was mutated to an adenine. This resulted in the codon for a leucine (CTG) being changed to a codon for a methionine (ATG) at position 113 of the proteins (L113M).

To better understand how these point mutations may affect the interactions of the alpha and beta subunits of *P.aerugionsa* PheRS and where the mutated amino acids occur relative to the functional areas of the protein, we aligned amino acids of *P. aeruginosa* PheRS alpha-subunit with *E.* coli PheRs alpha-subunit (Figure 22) and modeled these mutations onto the 3D structure of the alpha-subunit from *E.* coli (PDB 3PCO) (Figure 23). This model was then used to identify

the location of the mutated amino acids on the 3D structure of the *P. aeruginosa* PheRS apo form (figure 24).

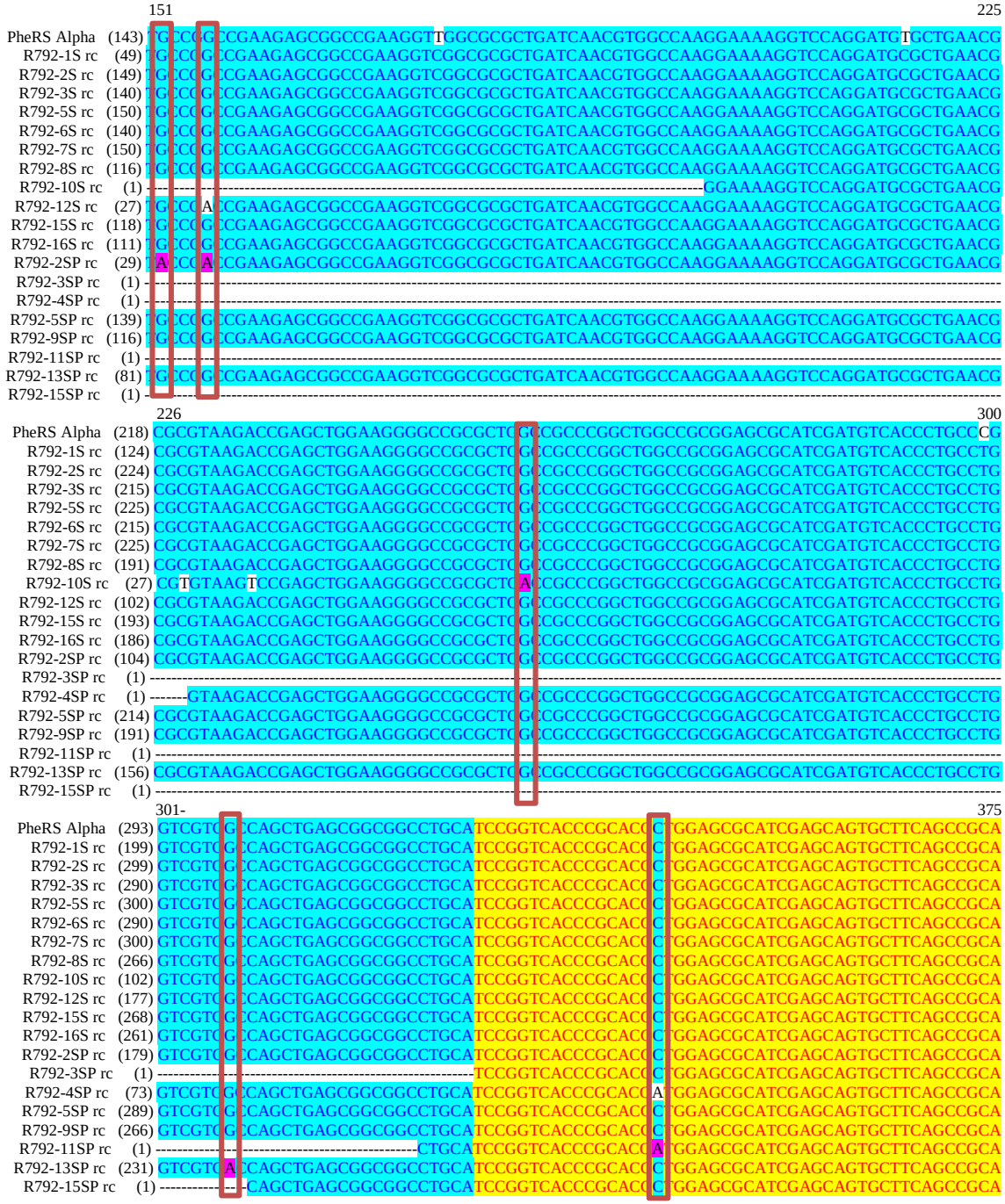


Figure 21. Alignment of nineteen samples from spontaneous and serial passage mutant generation using primer R792 in comparison to the native form of the gene encoding the PheRS alpha subunit. Mutation of nucleotide sequence is depicted by magenta outlined by red box. Sequence variations not shown in boxes are artifacts of PCR.

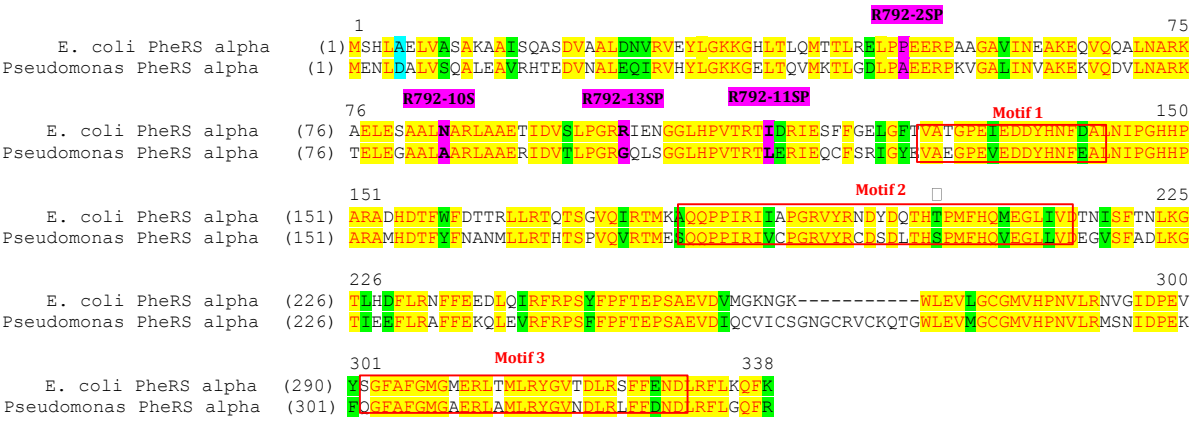


Figure 22. Amino acid alignment of *P. aeruginosas* PheRS alpha subunit with *E. coli* PheRS alpha subunit. The location of mutated amino acids are depicted by the magenta color. Motifs 1, 2 and 3 are outline with a red box.

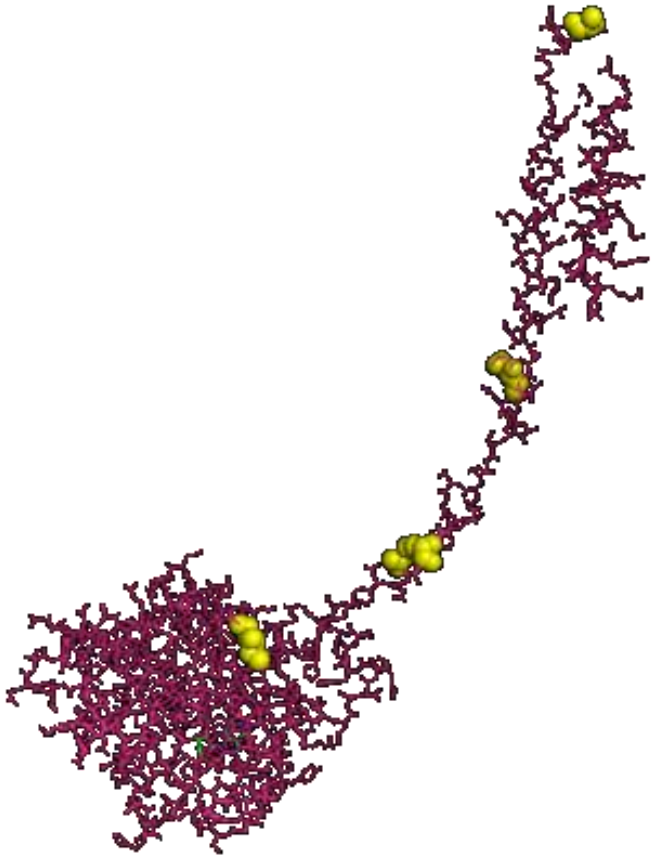


Figure 23. 3D structure of *E.coli* PheRS alpha subunit (PDB 3PCO) with mutated amino acids shown in yellow. The location of the mutated amino acids are determined from an alignment with *P. aeruginosa* PheRS alpha subunit.

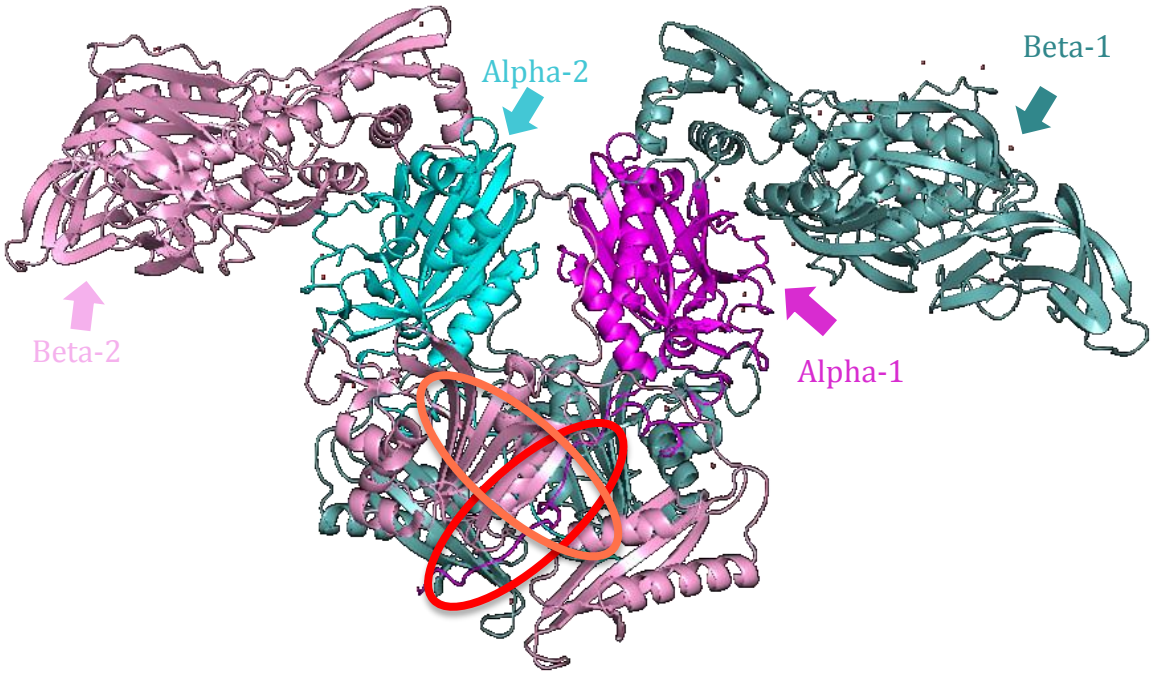


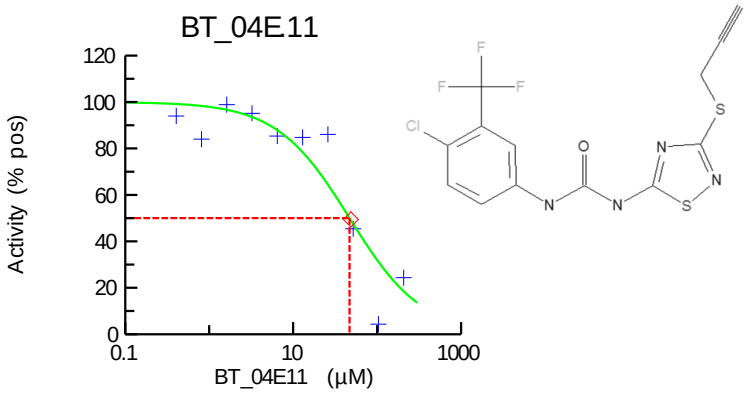
Figure 24. 3D structure of *P. aeruginosa* PheRS apo structure. The Alpha and Beta subunits are labeled. Approximate positions of the mutated amino acids are designated by red oval corresponding to Alpha-1 and orange oval corresponding to Alpha-2.

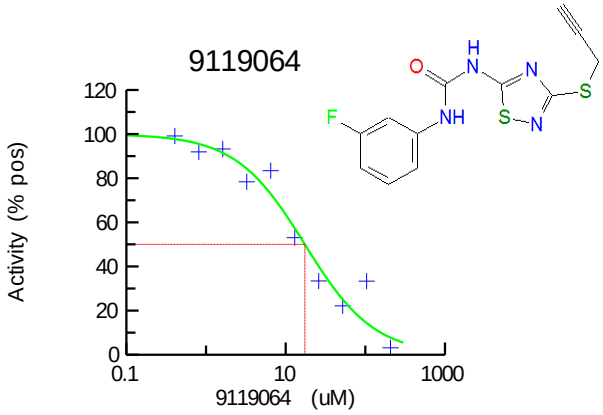
Structural Activity Relationship (SAR) Development

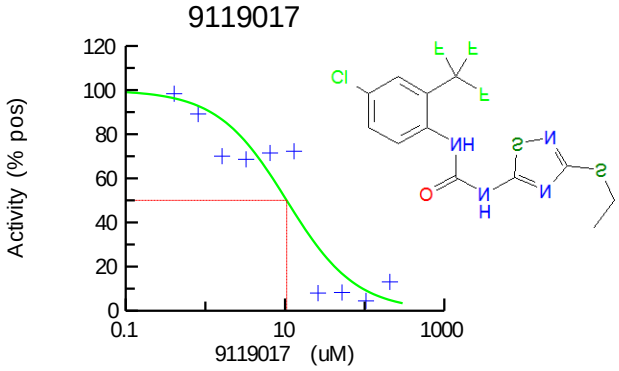
The structures of the four lead compounds (BT_04E11, BT_06G06, BT_06D07 and BT_08F11) were used to identify 16 derivatives or structure activity relationship (SAR) compounds (Ambinter chemical compound datatbase). To determine the effects of modifying regions of the original compounds on the inhibitory activity values, IC_{50} (Figure 25 and 26) and MIC (Table 5) were determined as described in 'Materials and Methods'.

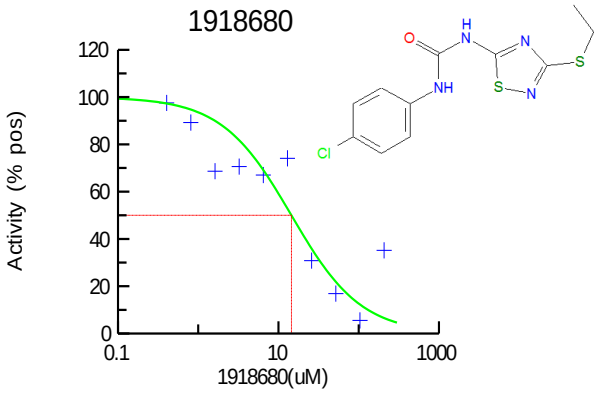
IC_{50} data was analyzed using XL fit (version 5.1;IDBS) as part of Microsoft Excel. IC_{50}'s using the A/T assays for the 16 SAR chemical compounds ranged from 5.7 μM to 67.1 μM (Figure 25). IC_{50}'s in the aminoacylation assays for 8 SAR chemical compounds that were shown to target PheRS ranged from 26.9 μM to 96.5 μM (Figure 26). The IC_{20} values for the 16 chemical compounds are shown in table 4.

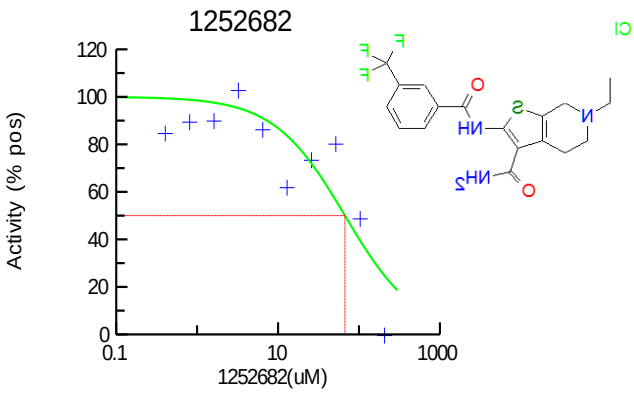
BT_04E11 Derivatives (A/T assay):



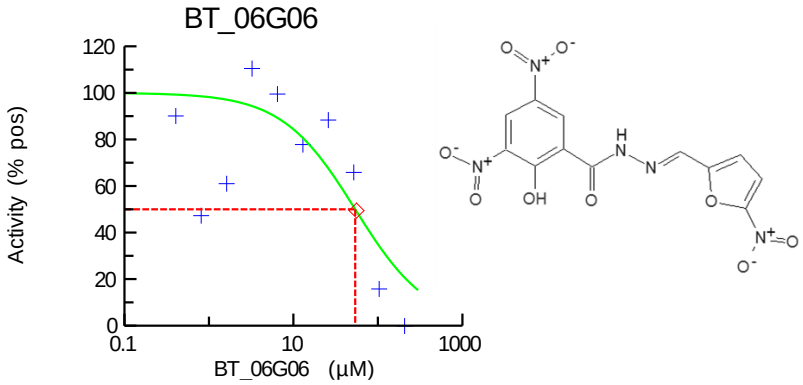


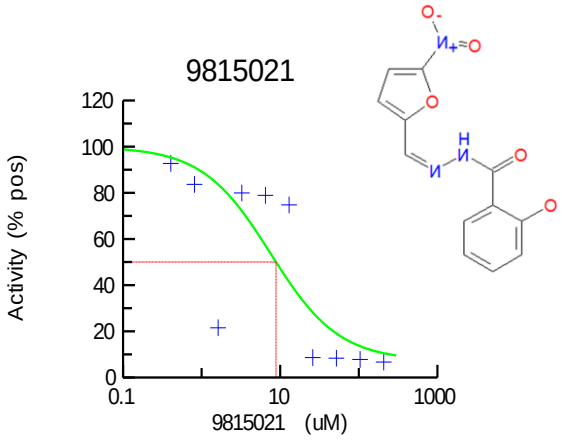


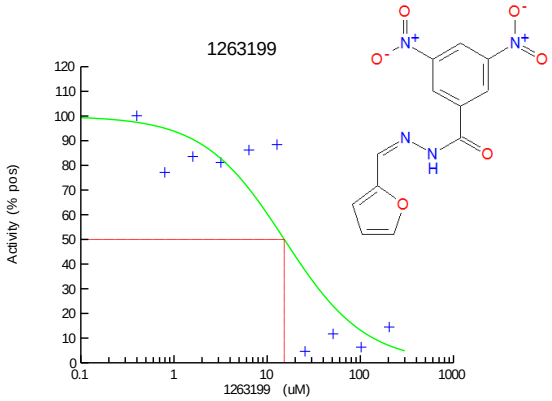


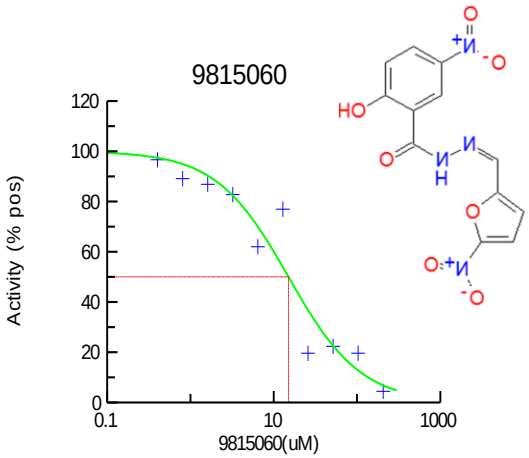


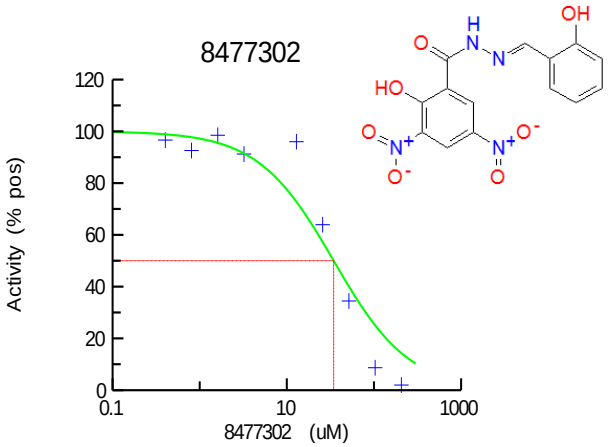
BT_06G06 Derivatives (A/T assay):



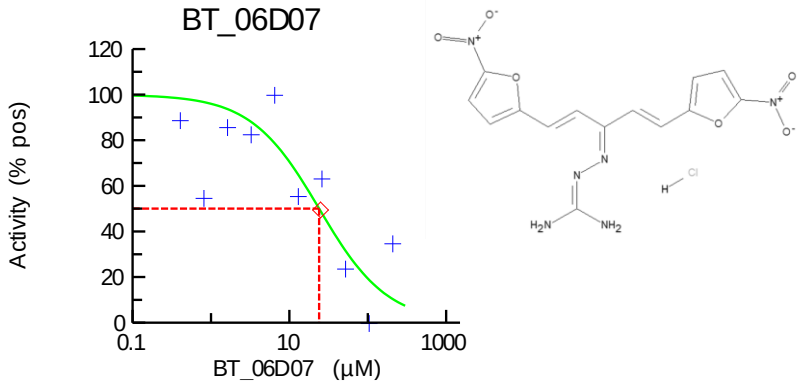


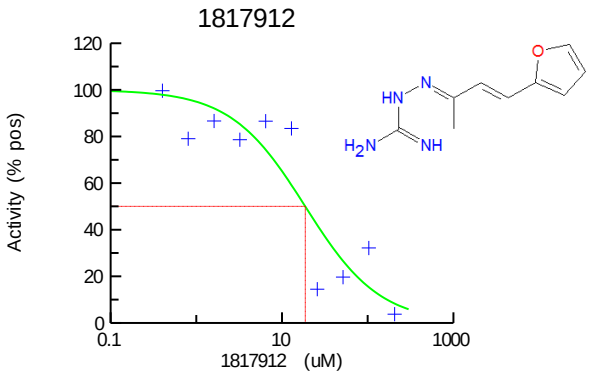


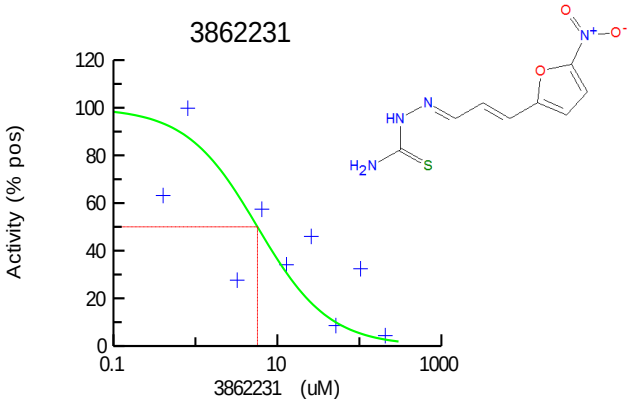


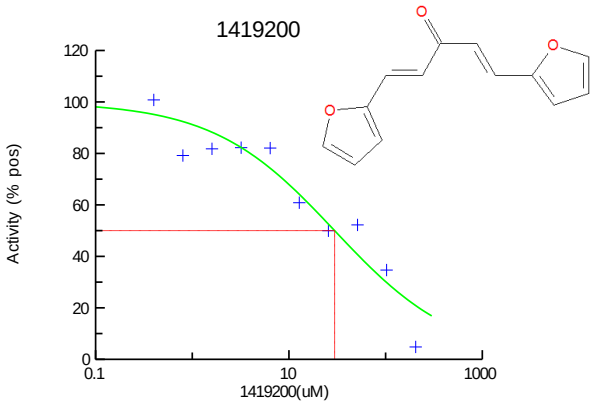


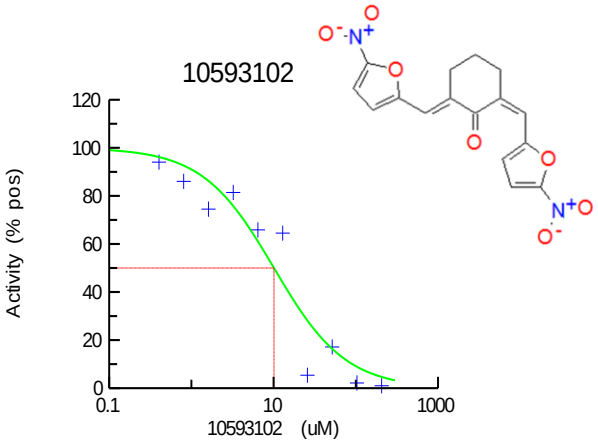
BT_06D07 Derivatives (A/T assay):











BT_08F11 Derivatives (A/T assay):

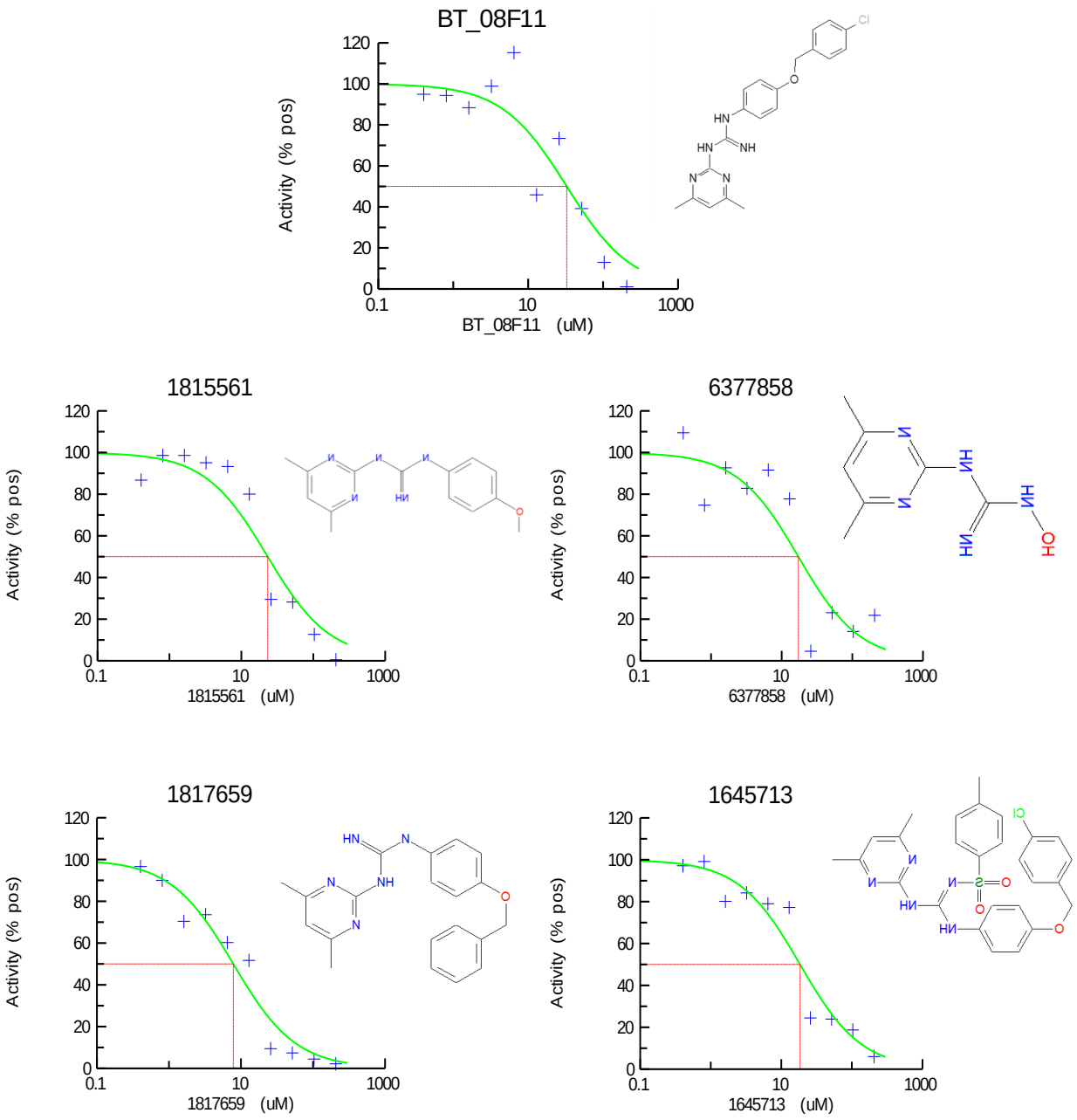
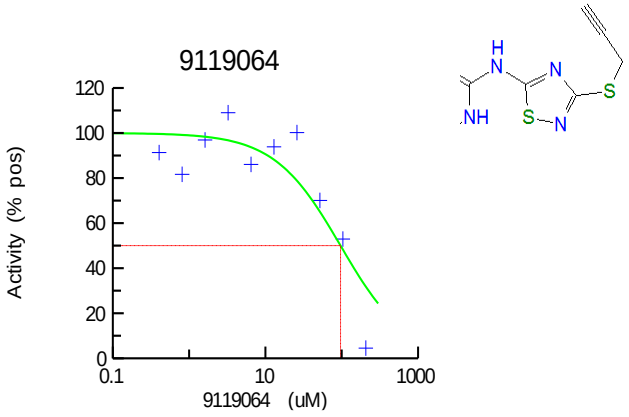
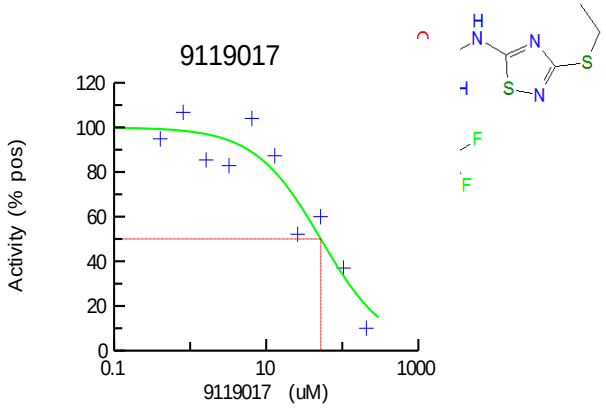
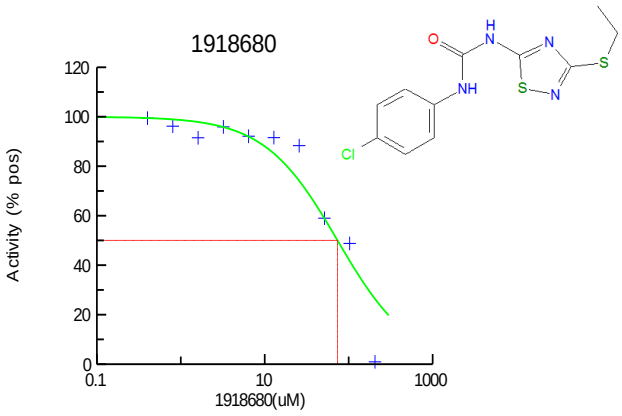


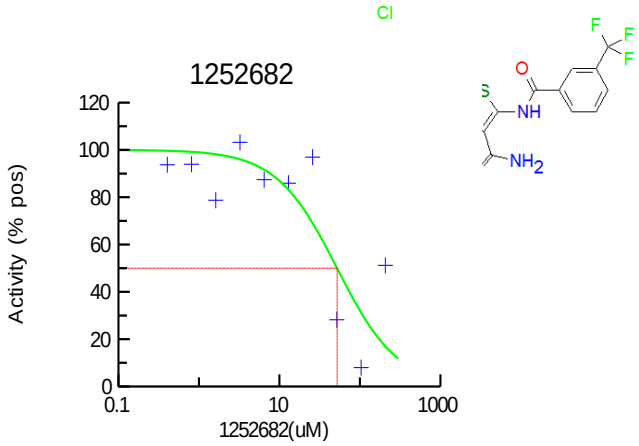
Figure 25. IC_{50} results of SAR compounds in comparison to parent structure along with structures. IC_{50}'s were determined using the A/T assay.

BT_04E11 Derivatives (Aminoacylation Assay):









BT_06G06 Derivatives (Aminoacylation Assay):

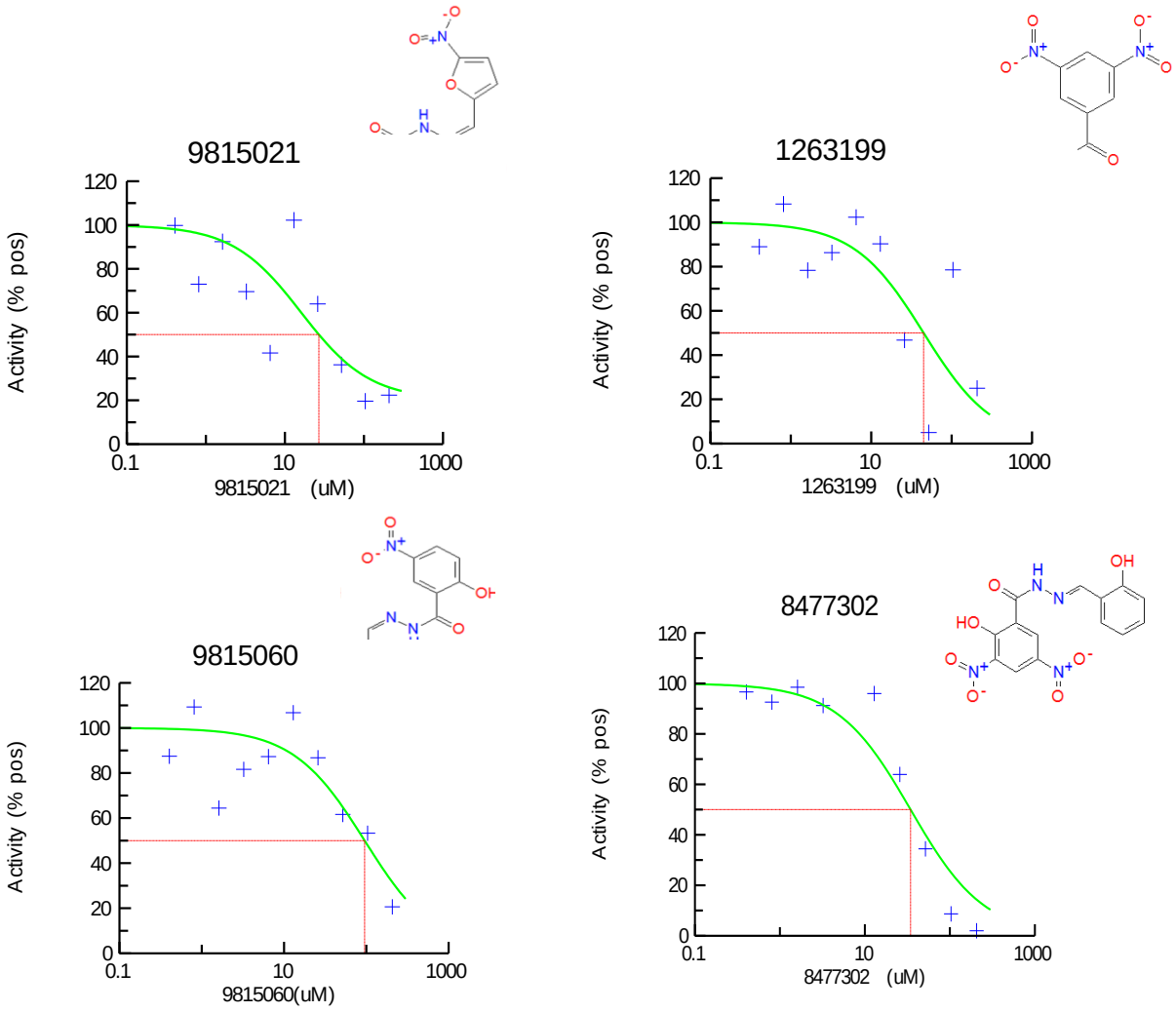


Figure 26. IC_{50} results of SAR compounds derived from parent compounds that target PheRS determined using aminoacylation assay.

Chemical Compounds	IC_{50} Value	
	A/T	Amino-acylation
BT_04E11	**47.0**	
Amb9119064	17.4	96.5
Amb9119017	10.5	52.1
Amb1918680	14.5	73.6
Amb1252682	67.1	52.1
BT_06G06	**53.9**	
Amb9815021	8.8	26.9
Amb1263199	15.3	44.8
Amb9815060	15.0	95.5
Amb8477302	34.5	63.8
BT_06D07	**24.0**	
Amb1817912	18.7	N/A
Amb3862231	5.7	N/A
Amb1419200	29.7	N/A
Amb10593102	10.1	N/A
BT_08F11	**32.8**	
Amb1815561	23.3	N/A
Amb6377858	17.2	N/A
Amb1817659	7.8	N/A
Amb1645713	18.5	N/A

Table. 4. Comparison of IC_{50} values of parent structure and the corresponding SAR derivatives. IC_{50} values were determined using the A/T assay for the parent and SAR derivatives and the aminoacylation assay for BT_04E11 and BT_06G06 corresponding derivatives that target PheRS.

MIC Value (µg/mL)										
Chemical Compound	**M. cat**	**H. flu**	**E. coli**	**S. aureus**	**S. pneumo**	**E. faecalis**	**E.coliTolC Efflux**	**P. aeruginosa**	**P.aeruginosa Hypersensitive**	**P.aeruginosa PA200Efflux**
BT_04E11	128	2	128	1	8	128	0.5	2	16	128
Amb9119064	128	16	>128	128	16	16	2	64	16	32
Amb9119017	128	8	>128	128	16	64	1	32	16	128
Amb1252682	>128	128	>128	>128	32	>128	128	128	128	128
Amb1918680	128	64	>128	128	16	64	>128	128	64	128
BT_06G06	32	32	128	32	128	128	2	16	128	128
Amb9815021	>128	128	128	128	128	128	32	128	128	128
Amb9815060	128	64	>128	64	128	128	32	128	64	128
Amb1263199	128	>128	>128	>128	32	128	128	>128	128	64
Amb8477302	128	32	>128	128	8	>128	128	128	128	128
BT_06D07	64	16	128	16	32	128	2	64	32	128
Amb1817912	>128	128	>128	>128	32	128	>128	128	128	32
Amb3862231	16	16	32	16	64	16	4	128	4	32
Amb1419200	>128	>128	>128	128	>128	128	>128	128	128	128
Amb10593102	64	32	>128	32	64	32	128	128	64	128
BT_08F11	64	32	64	32	32	16	16	64	128	32
Amb1815561	>128	>128	>128	>128	32	128	>128	>128	128	32
Amb6377858	>128	128	>128	128	128	128	>128	128	128	64
Amb1817659	64	32	128	64	32	64	32	16	16	64
Amb1645713	>128	>128	>128	>128	128	128	>128	>128	128	128

Table 5. MIC results of parent compound and the corresponding 16 SAR derivatives against a panel of 10 pathogenic bacteria.

CHAPTER V

CONCLUSION

Having a previously developed poly(U) mRNA directed (A/T system) protein synthesis system form *Pseudomonas aeruginosa*, this system was used to screen a synthetic chemical compound library compose of 890 compounds (TimTec) to identify inhibition of the system. 20 compounds were identified to inhibit greater than 50% of the activity of the A/T system: three compounds (BT_01D11, BT_04E11, and BT_06G06) inhibited the activity of PheRS, one compound (BT_05H05) inhibited EF-Tu, and sixteen compounds (BT_01B11, BT_02A08. BT_03F03, BT_04D03, BT_05B02, BT_05H09, BT_06D07, BT_07D07, BT_08F06, BT_08F11, BT_09F04, BT_09G04, BT_09B10, BT_10F04, BT_10A10, and BT_10C11) inhibited the ribosomal activity.

Microbiological assays consisted of broth micro-dilution minimal inhibitory concentration (MIC) testing and time-kill kinetic studies. MIC results showed that four compounds (BT_04E11, BT_06G06, BT_06D07 and BT_08F11) had overall low MIC values against most of the pathogenic bacteria tested. Using these four compounds in time-kill studies, compound BT_06D07 showed bactericidal activity against both *H. influenzae* and *S. aureus*, while compound BT_06G06 was bacteriostatic against both of these pathogens. Compound BT_04E11 was bacteriostatic against *H. influenza*, but results indicated it was bactericidal as a mode of inhibitory action against *S. aureus*. Compound BT_08F11 is bacteriostatic against both *H. influenzae* and *S. aureus* but at higher concentrations complete inhibition could be observed.

The binding mode of BT_04E11 and BT_06G06 were studied to determine the effects of potential competition of the compounds with the natural substrates of PheRS (ATP and Phe). Results showed that compound BT_04E11 was competitive with ATP and noncompetitve with Phe. CompoundBT_06G06 was determined to be noncompetitive with both ATP and Phe.

Since both BT_04E11 and BT_06G06 inhibited bacterial PheRS, these two compounds were tested to determine possible effects against human mitochondrial PheRS (hmPheRS). Results showed neither compound inhibited the activity of hmPheRS. All four compounds were then used to determine potential inhibition on cytosolic protein synthesis system using a wheat germ cell extract. There was no inhibitory effect observed for any of the compounds on eukaryotic cytoplasmic protein synthesis. All four compounds were then tested to determine cytotoxicity in mammalian cells. Compound BT_04E11 showed no level of toxicity at any concentration tested. BT_06D07 observed toxicity at 2000-fold higher than staurosporin (positive control) and both compounds BT_06G06 and BT_08F11 were observed to be toxic at approximately 500-fold higher than the positive control.

To provide evidence that the four inhibitory compounds (BT_04E11, BT_06G06, BT_06D07 and BT_08F11) in fact did inhibit protein synthesis in *P. aeruginosa* hypersensitive, macromolecular synthesis assays were used. Compounds BT_04E11 and BT_06G06 demonstrated a dose-dependent inhibition of protein, RNA, DNA, and cell-wall synthesis, as was expected with compounds that elicit the stringent response. While compound BT_06D07 and BT_08F11 showed primary inhibitory effects on protein synthesis in comparison to the other three macro-molecular components.

Compound BT_04E11 was used for the generation of mutants in *P.aeruginosa* hypersensitive. Spontaneous resistance and serial passage mutants developed a total of four

genetic level point mutations. Modeling of the amino acid mutations detected in the native form of *P. aeruginosa* was compared to *Escherichia coli* PheRS alpha subunit.

Four lead compounds (BT_04E11, BT_06G06, BT_06D07, and BT_08F11) were used as the parent structures in the development of 16 structural activity relationship compounds. IC_{50}'s and MIC's of the SAR compounds were compared to parent structure for identification of modification leading to a potential increase of potency.

In conclusion, the 4 lead compounds represent a promising narrow spectrum, new mode-of action agents with excellent microbiological profiles that warrant its further evaluation as a novel drug to combat *P. aeruginosa* infections.

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APPENDIX A

APPENDIX A

Plate map of chemical compound screening, and media components in MIC and time-kill study.

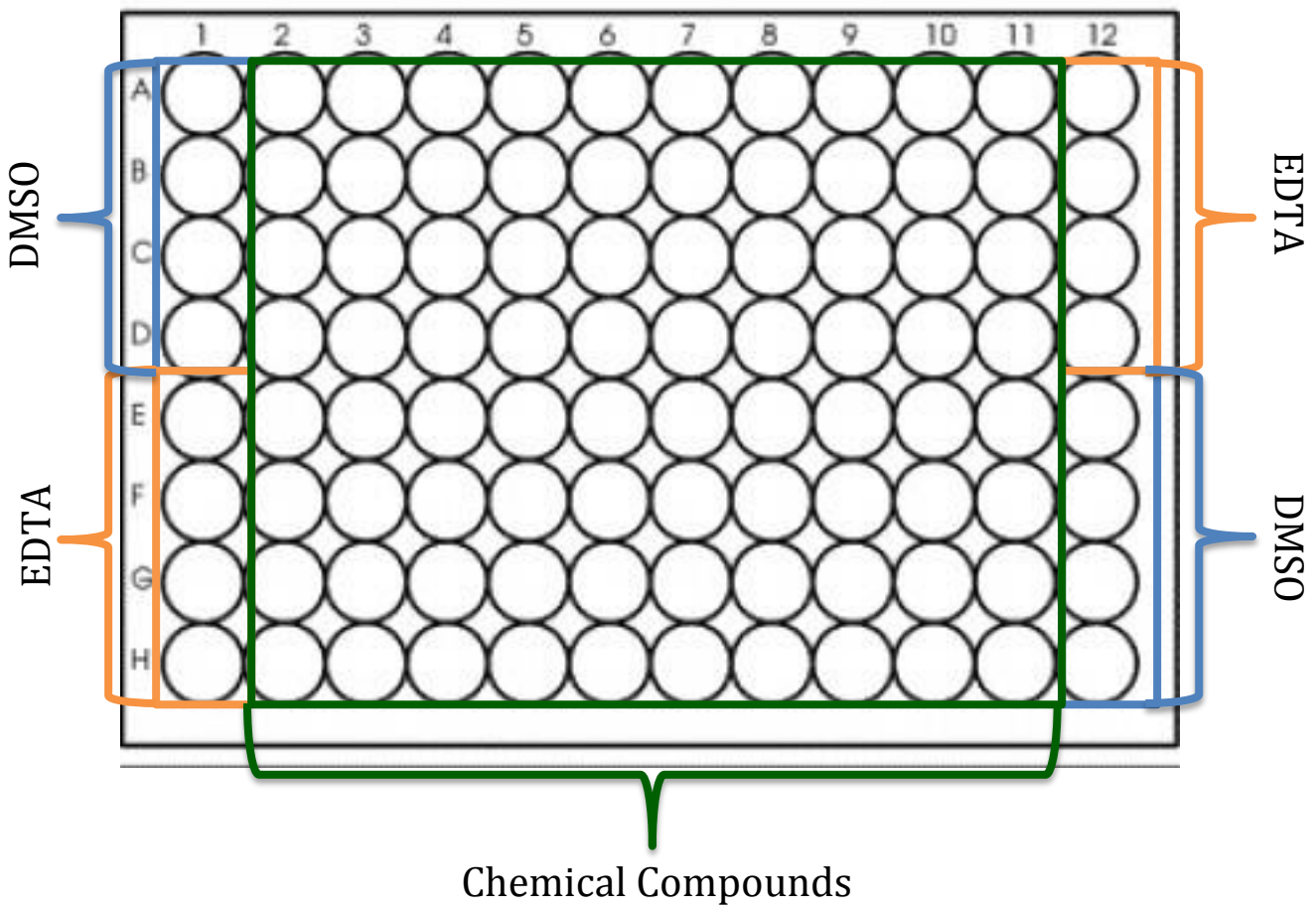

Figure 27. Composition of a 96 well microtiter plate used in chemical compound screening.

Bacteria	Liquid Media	Solid Media
M. catarrhalis	Brain Heart Infusion	Trypicase Soy Broth w/3% blood
H. influenzae	Haemophilus Test Medium	Chocolate Agar Plate
E. faecalis	Brain Heart Infusion	Trypicase Soy Broth w/3% blood
S. pneumoniae	Brain Heart Infusion w/ 3% blood	Trypicase Soy Broth w/3% blood
E. coli	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood
E. coli TolC Efflux	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood
S. aureus	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood
P. aeruginosa	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood
P. aeruginosa Hypersensitive strain	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood
P. aeruginosa PA200 Effflux	Trypticase Soy Broth	Trypicase Soy Broth w/3% blood

Table 6. Liquid and solid Medias used for bacterial growths in MIC and time-kill studies.

Bacteria	QC Agent	QC Agent MIC Range
M. catarrhalis	0.16 mg/mL Ampicillin	0.12-0.5
H. influenzae	0.32mg/mL Ampicillin	0.12-0.5
E. faecalis	1.28 mg/mL Vancomycin	2.0-8.0
S. pneumoniae	0.64 mg/mL Penicillin	0.25-1.0
E. coli	1.28 mg/mL Ampicillin	2.0-8.0
E. coli TolC Efflux	1.28 mg/mL Ampicillin	2.0-8.0
S. aureus	0.32 mg/mL Oxycline	0.12-0.5
P. aeruginosa	1.28 mg/mL Tetracycline	0.12-1.0
P. aeruginosa Hypersensitive strain	1.28 mg/mL Tetracycline	0.12-1.0
P. aeruginosa PA200 Effflux	1.28 mg/mL Tetracycline	0.12-1.0

Table 7. Quality control (QC) reagents. QC agents and QC agent MIC Range shown for the panel of 10 pathogenic bacteria used in MIC testing.

++ Media
200 mL M_9 salts (1.2 g Na_2HPO_4, 0.6 g KH_2PO_4, 0.2 g NH_4Cl)
50 mL (1M) MOPs
1.0 g NH_4Cl
1.0 mL 40% glucose
200 μL (1M) MgOAc
3.5 g Trypticase Soy Broth
+ H_20
1 Liter of Media

Table 8. Components that make up 1 liter of ++ media used for MMS Assay.

APPENDIX B

APPENDIX B

Protocol of all the assays used in this project:

1.) Initial screening of chemical compound library using the A/T system:

a) To make the working plates, dilute the parent plates of the chemical compound library (10mM) to the concentration of 3.3 mM with DMSO.

b) 2 μL of DMSO, EDTA (0.5 M pH 8.0), or chemical compounds (3.3 mM) were added into a 96 well microtiter plates (Costar) as indicated in figure 27.

c) Master mix was made as shown below:

Master Mix	**Stock Concentration**	**Final concentration**	**μL/Rx**
H_2O			15.33
P. aeruginosa ribosomes	6.0 μ M	0.2 μM	1.67
Tris-HCl (pH 7.5)	1.0 M	50 mM	1.67
$MgCl_2$	1.0 M	10 mM	0.5
KCl	1.0 M	25 mM	1.25
PK	12.5 Units/μL	0.025 Unites/μL	0.1
PEP	100 mM	4 mM	2.0
ATP	100 mM	1.5 mM	0.75
GTP	100 mM	0.5 mM	0.25
Spermine	10 mM	0.03 mM	0.15
DTT	1.0 M	1 mM	0.05
[^{3}H] Phenylalanine (75cpm/pmol)	1250 μM	40 μM	1.6
Poly(U) mRNA	5.0 mg/mL	0.3 mg/mL	3.0
P. aeruginosa EF-Tu	18.9 μM	1.0 μM	2.65
P. aeruginosa EF-G	15.5 μM	0.2 μM	0.65
P. aeruginosa EF-Ts	243 μM	0.05 μM	0.01
P. aeruginosa PheRS	7.04 μM	0.075 μM	0.54
Total			33

d) 33 μL of master mix was added to each well of the microtiter plate and incubated at room temperature for 15 min to allow interaction of the enzymatic components with the compounds, DMSO or EDTA.

e) 15 μL of *E. coli* tRNA solution (3 μL of 416 μM tRNA stock and 12 μL H_2O, tinto each well of the microtiter plate in order to start the reaction. Reaction was incubated for 1 h at 37 °C.

f) Reaction is stopped by the addition of 5 μL of 0.5 M EDTA (pH 8.0).

g) 200 μg SPA beads solution (148 μL of 300 mM pH 6.2 citrate buffer and 2 μL of 100 mg/mL of RNA binding beads [YSI]; Perkin Elmer) was added into each well.

h) Microtiter plates were left to settle for 1 h at room temperature.

i) Plates were read using 1450 Microbeta Jet Liquid Scintillation and Luminescence Counter (Wallac)

2.) Confirmation Screening of Primary Hits:

a) Steps a-i from initial screening are used in duplicate form to confirm initial compound hits.

3.) IC_{50} Assay of Chemical Compound Library Confirmed Hits:

a) Chemical compounds were serially diluted in DMSO for concentration of: 5, 2.5, 1.25, 0.625, 0.3125, 0,15625, 0.078125, 0.0390625, 0.01953125, and 0.009765625 mM.

b) 2 μL of diluted compound was add into a 96 well microtiter plate, which would make the concentration of the compound in the assay at: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, and 0.390625 μM; 2 μL of DMSO and EDTA were as well added to specified wells as indicated in figure 27.

c) Steps c-i were followed as described in initial/ confirmational screening.

4.) Target determination:

I.) PheRS Aminoacylation Assay:

a) 2 µL of 0.5 M EDTA (pH 8.0), DMSO or confirmed chemical compounds (3.3 mM) were added into 96 well microtiter plate.

b) Master mix was made as shown below:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			24.75
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
$MgCl_2$	1.0 M	8.0 mM	0.4
ATP	100 mM	2.5 mM	1.25
Spermine	100 mM	1.0 mM	0.5
DTT	1.0 M	1 mM	0.05
[^{3}H] Phenylalanine (75cpm/pmol)	1250 µM	75 µM	3.0
P. aeruginosa PheRS	7.04 µM	0.075 µM	0.55
Total			33

c) Steps d-f were followed as described in initial/confirmational screening.

d) 400 µg beads solution (146 µL of 300 mM pH 2.0 citrite buffer and 4 µL of 100 mg/mL of Poly-Lysine coated beads [YSI]; Perkin-Elmer) was added into each well.

e) Plates were read using 1450 Microbeta Jet Liquid Scintillation and Luminescence Counter (Wallac)

II.) EF-Tu and EF-Ts GDP Exchange Assay:

a) 2 µL of 0.5 M EDTA (pH 8.0), DMSO or confirmed chemical compounds (3.3 mM) were added into glass tubes and set on ice.

b) Master mix was made as shown below:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			33.8
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
MgCl2	1.0 M	10 mM	0.5
NH4Cl	1.0M	50 mM	2.5
DTT	1.0 M	1 mM	0.05
P. aeruginosa EF-Tu	18.9 µM	1.0 µM	2.65
Total			42

c) 42 µL of master mix was added into the glass tubes

d) Reaction was incubated for 15 min at room temperature.

e) Reaction was started by the addition of 6 µL of 100 µM [^{3}H] GDP.

f) Reactions was incubated for 30 min in a 37 °C water bath.

g) Reaction was stopped by the adding of 1.0 mL of ice cold wash buffer (50 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$), and placed on ice.

h) Reaction solutions were filtered through nitro-cellulose filter, which had been previously soaked in 5%TCA. Filters were washed 3 times with 2 mL portions of wash buffer.

i) Filters were dried and counted using a Perkin-Elmer scintillation counter.

III.) EF-G GTPase Assay:

a) 2 µL of 0.5 M EDTA (pH 8.0), DMSO or confirmed chemical compounds (3.3 mM) were added into 96 well plate.

b) Master mix was made as shown below:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			30.13
P. aeruginosa ribosomes	6.0 µ M	0.2 µM	1.67
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
$MgCl_2$	1.0 M	10 mM	0.5
NH_4Cl	1.0M	70 mM	3.5
DTT	1.0 M	1 mM	0.05
P. aeruginosa EF-G	15.5 µM	0.2 µM	0.65
Total			39

c) 39 µL of master mix was added into each well in the plate and incubated at room temperature for 15 min.

d) Reaction was started by the addition of 9 µL of 10 mM GTP.

e) Reaction was incubated for 30 min in a 37 °C heat block.

f) Reaction was stopped by the addition of 150 µL of 50 mM EDTA.

g) 50 µL of Gold Lock Mix (50 µL of Gold Lock and 0.5 µL of Accelerator) was added into each well.

h) Plate was set at room temperature for 20 min followed by reading the absorbance at 595 nm(OD_{595}) using Bradford method using Coomassie Protein Assays Reagents (Thermo Scientific) Multiscan software

IV.) LeuRS Aminoacylation Assay:

a) 2 µL of 0.5 M EDTA (pH 8.0), DMSO or confirmed chemical compounds (3.3 mM) were added into 96 well microtiter plate

b) Master mix was made as shown below:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			25.11
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
MgOAC	1.0 M	5.0 mM	0.25
ATP	100 mM	2.5 mM	1.25
[^{3}H] Leucine (75cpm/pmol)	1250 µM	75 µM	3.0
P. aeruginosa LeuRS	5.64 µM	0.1 µM	0.89
Total			33

c) Steps c-e were followed as decribed under PheRS assay.

5.) MIC Testing:

a) Bacteria is grown on fresh agar plate a day prior to MIC testing (bacteria and media along with agar are shown in table 6).

b) 15 mL of broth growth medium is pre-warmed to room temperature

c) Media from step b was inoculated with 3-5 colonies from the agar plate in step a, and grown in a 37 °C incubator at 200 rpm for 2 to 6 h or until OD_{625} reaches 0.08 to 0.13.

d) If needed, bacterial growth from step c was diluted to the OD_{625} range of 0.08 to 0.13.

e) Prepare microtiter plate: 90 µL of growth medium from step d was added into wells of column1 and 50 µL of growth medium was added into wells in columns 2 through 12.

f) 10 µL of test compounds (1.28 mg/mL) were added to the first column. The last column contained QC agent (Bacteria with QC agent and QC MIC range shown in table 7).

g) Compound was serially diluted by transferring 50 µL medium from column 1 into column 2and mixed well. Serial dilutions were continued to column 11.

h) After mixing well contents in column 11, 50 µL were discarded.

i) Plates were incubated at 37 °C.

j) MIC results were read after 24 h incubation.

6.) Time-Kill Study:

a) Bacteria is grown in media a day prior to time kill study (refer to table 6 for liquid and solid media used).

b) 10 mL of liquid medium was inoculated with 0.1 mL of overnight bacterial growth from step a, and grown in a 37 °C incubator at 200 rpm for 2 h or until its OD_{625} reaches 0.08 to 0.13.

c) Two 10 mL of medium flask are prepared as followed and pre-warmed to 37 °C:

   i. Medium along with control (DMSO)

   ii. Medium along with chemical compound at 4 × MIC

d) Microtiter plates are prepared for sample dilution: 90 µL of 0.85% of sterile saline solution was added to column 2-8, leaving column 1 empty.

e) Bacterial growth from step b was diluted to the OD_{625} range of 0.08 to 0.13 by adding culture to 5 mL of 0.85% sterile saline solution until OD_{625} range is matched.

f) 0.1 mL of subculture from step e was used to inoculate flasks from step c.

g) Immediately following inoculations, 100 µL of sample from the freshly inoculated flasks was transferred into the first well in the microtiter plates that was described in step d, this is to be labeled as 0 h.

h) Start the timer after inoculation of media in step f, and incubate culture flasks at 37 °C in the incubator at 200 rpm.

i) Serially dilute microtiter plates from step g by transferring 10 µL of sample from column 1 into column 2 and mixed well.

j) 10 μL of sample from column 2 is transferred into column 3 and mixed well.

k) Serial dilutions were continued up to column 8 for concentrations of: 10e02, 10e03, 10e04, 10e05, 10e06, 10e07, 10e08, and 10e09.

l) Working row by row, 10 μL of sample from microtiter dilution plate was used to spot onto an agar plate subdivided into eight zones.

m) Plates were air dried until spots were soaked into plate followed by incubation at 37 °C.

n) Repeat microtiter plate preparation (step d) and remove serial dilution of microtiter plate (steps g-m) at 2, 4, 6, and 24 h post inoculation.

o) Colonies were enumerated the following day and CFU/mL were calculated (multiply colony number by serial dilution).

7.) Binding Mode Assay:

I.) ATP Competitive Assay:

a) Chemical compounds that were determined to inhibit tRNA synthetase were serially diluted as described in step a under IC_{50} assay

b) 2 μL of diluted compound was add into a 96 well microtiter plate, which would make the concentration of the compound in the assay at: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, and 0.390625 μM; 2 μL of DMSO and EDTA were as well added to specified wells as indicated in figure 27.

c) Master mix was made as shown below:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			27.00/26.99/26.97/26.9/ 26.77/26.52
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
$MgCl_2$	1.0 M	7.5 mM	0.4
ATP	100 mM	25/50/100/250/ 500/1000 µM	0.0125/0.025/0.05/0.125/ 0.25/0.5
Spermine	100 mM	1.0 mM	0.5
DTT	1.0 M	1 mM	0.05
[^{3}H] Phenylalanine (75cpm/pmol)	1250 µM	50 µM	2.0
P. aeruginosa PheRS	7.04 µM	0.075 µM	0.53
Total			33

d) Varying concentrations are shown along with appropriate amounts of water and ATP added in µL/Rx (i.e. for 25 µM ATP, 0.0125 µL/Rx of ATP is added and 27.00 µL/Rx was added to the master mix)

e) Steps c-d were followed as described under PheRS Assay.

II.) Phe Competitive Assay:

a) Steps a-e from ATP competitive assay were followed with the exception of using the following Master mix:

Master Mix	Stock Concentration	Final concentration	µL/Rx
H2O			26.77/25.77/23.77/19.77/ 15.77
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
$MgCl_2$	1.0 M	7.5 mM	0.4
ATP	100 mM	2.5 mM	1.25
Spermine	100 mM	1.0 mM	0.5
DTT	1.0 M	1 mM	0.05
[^{3}H] Phenylalanine (75cpm/pmol)	1250 µM	25/50/100/200/ 300 µM	1.0/2.0/4.0/8.0/ 12.0
P. aeruginosa PheRS	7.04 µM	0.075 µM	0.53
Total			33

b) Varying concentrations are shown along with appropriate amounts of water and Phe added in µL/rxn (i.e. for 25 µM Phe, 1.0 µL/Rx of Phe is added and 26.77 µL/rxn was added to the master mix).

8.) Eukaryotic Protein Synthesis Assays:

I.) Human Mitochondrial PheRS Assay:

a) Chemical compound that inhibited the bacterial phenylalanine tRNA synthetase was diluted to: 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625 and 0.01953125 mM with DMSO.

b) 2 µL of diluted compound was added into glass tubes, to make concentration of the compound in the assay at: 200, 100, 50, 225, 12.5, 6.25, 3.125, 1.5625, and 0.78125 µM; 2 µL of DMSO or EDTA were added to glass tubes.

c.) Master mix was made as shown below:

Master Mix	**Stock Concentration**	**Final concentration**	**µL/Rx**
H2O			24.65
Tris-HCl (pH 7.5)	1.0 M	50 mM	2.5
MgOAc	1.0 M	10.0 mM	0.5
ATP	100 mM	2.5 mM	1.25
Spermine	100 mM	1.0 mM	0.5
DTT	1.0 M	1 mM	0.05
[^{3}H] Phenylalanine (75cpm/pmol)	1250 µM	75 µM	3.0
human mitochondrial PheRS	46 µM	0.5 µM	0.55
Total			33

d.) 33 µL of master mix was added to each glass tube and set to incubate at room temperature for 15 min.

e.) To start the reaction, 15 µL *E. coli* tRNA solution (9 µL of 668 µM tRNA stock and 6 µL of water.

f.) Glass tube reactions were incubate in a 37 °C waterbath for 1 h.

g.) Reactions were stopped by the addition of 2 mL of 5% TCA

j) Reaction solution were filtered through Whatman glass-fiber filters, which had been previously soaked in 5%TCA. Filters were washed 2 times with 5 mL portions of 5% TCA

k) Filters were dried and counted using a Perkin-Elmer scintillation counter.

II.) Wheat Germ Extract Assay:

a) Steps a and b were followed as described in Human Mitochondrial PheRS Assay.

b) Master mix was made as shown below:

Master Mix	**Stock Concentration**	**Final concentration**	**µL/Rx**
H2O			10.7
Poly(U)RNA	5 mg/mL	3 mg/mL	3.0
MgOAc	1.0 M	6.0 mM	0.3
[^{3}H] Phenylalanine (100cpm/pmol)	1250 µM	75 µM	3.0
Wheat Germ Extract			30.0
Yeast tRNA-Phe	200 µM	4.0 µM	1.0
Total			48

c) 48 µL of master mix was added to each glass tube and incubated in a 37 °C water bath for 1 h.

d) Reactions were stopped by the addition of 2 mL of 5% TCA.

e) Reactions were placed in a 90 °C heater block for 10-15 min.

f) Reaction solution were filtered through Whatman glass-fiber filters, which had been previously soaked in 5%TCA. Filters were washed 2 times with 5 mL portions of 5% TCA

g) Filters were dried and counted using a Perkin-Elmer scintillation counter.

9.) In-vitro Cytotoxicity Test:

a) Compound was dissolved to 12.8 mg/mL.

b) Human cells were capable of uptaking compound at 6% DMSO; therefore highest concentration of compound in assay must be 800 μg/mL.

c) Compound was serially diluted with concentrations of: 800, 400, 300, 200, 100, 50 and 25 μg/mL.

d) Dr. Megan Keniry (UTRGV) took over the experimentation.

10.) Macro-molecular Synthesis (MMS) Assay:

a) Grow bacteria on fresh agar plate a day prior to MIC testing.

b) 15 mL of broth growth medium is pre-warmed to room temperature

c) Media from step 2 was inoculated with 3-5 colonies from the agar plate in step a, and incuated in a 37 °C incubator at 200 rpm for 2 to 6 h or until $OD_{600} > 1$.

d) Prepare four (Thymine, Leucine, Alanine, and Uracil) microtiter plate: 90 μL of ++ media (Table 8) was added into wells of column 1 and 50 μL of ++ medium was added into wells in columns 2 through 12.

   i. 10 μL of DMSO (control), chemical compounds (1.28 mg/mL), or standard drug [1.28 mg/mL Levofloxacin (Thymine), 1.28 mg/mL Tetracycline (Leucine), 1.28 mg/mLVancomycin (Alanine), and 0.02 mg/mL Rifampin (Uracil)] were added to first well of each column corresponding to the appropriate plate.

   ii. 50 μL from column 1 were transferred to column 2 and mixed well.

   iii. 50 μL from column 2 were transferred to column 3 and mixed well.

iv. Serial dilution was continuing down to column 12 and last 50 µL were discarded.

v. Plates were pre-warmed at 37 °C.

e) Isotopes were prepared by the addition of 50 µL of concentrated isotope into 1.2 mL of ++ media.

f) Bacterial growth from step c was harvested by centrifugation of cell (4,000 rpm for 5 min); all remnants of supernatant were removed.

g) Pellet was re-suspended in 0.5 mL of 0.85% saline solution.

h) Subculture from step g was used to determine dilution needed to reach McFarland standard in 5 mL of 0.85% saline solution.

i) In determining the dilution fold in step g, 1 mL of cells in saline solution was made at 30 × 0.5 McFarland.

j) Four 50 mL tubes are labeled either "Thymine, Leucine, Alanine, and Uracil", contained 5 mL of ++ media and the following bacterial growth from step i:

i. In the thymine pre-labeled tube, 200 µL bacterial growth was added

ii. In the leucine pre-labeled tube, 750 µL bacterial growth was added

iii. In the alanine pre-labeled tube, 400 µL bacterial growth was added

iv. In the uracil pre-labeled tube, 100 µL bacterial growth was added

k) In respectfully labeled microtiter plate, 50 µL of bacterial dilution from step j was transferred into each well of the plate and incubated for 20 min in a 37 °C heat block.

l) 10 µL of respectfully labeled isotope prep from step e, was added into each well of the microtiter plate and incubated for 20 min in a 37 °C heat block.

m) Reactions were stopped by the addition of 90 µL of ice cold 20% TCA.

n) Plates were placed in refrigerator for 1 h.

o) Samples were then filtered through 96 well Millipore plates using vacuum manifold.

p) Plate was washed twice with 200 μL of 10% TCA and once with 200 μL of cold ethanol.

q) Plates were dried overnight.

r) 100 μL of scintillation fluid was added to each well after sealing bottom of plates and plates were read using 1450 Microbeta Jet Liquid Scintillation and Luminescence Counter (Wallac).

11.) Mutant Generation:

II.) Spontaneous Resistance:

a) Grow bacteria on fresh agar plate a day prior.

b) 50 mL of trypticase soy broth was inoculated with a few colonies from step a and grown in a 37 °C incubator set at 200 rpm for 4 to 6 h or until $OD_{600} > 1$.

c) Bacterial cell were harvested by centrifugation (4,000 rpm, 10 min) and re-suspended in 1-2 mL of TSB broth.

d) Subculture from step c was used to determine dilution needed to reach 0.5 McFarland standard in 5 mL of 0.85% saline solution.

e) In determining the dilution fold in step d, cell suspension was prepared in TSB at 1×10^{10} cell per mL.

f) 0.1 mL of cell suspension was plated on an agar plate containing compound inhibitor at 4 × MIC.

g) Colonies were enumerated after 96 h.

h) If mixed colony morphotypes are present, individual colonies are re-streaked onto fresh blood agar plates.

i) Colonies were picked and individually inoculated into 3 mL of TSB and grown in a 37 °C incubator at 200 rpm overnight.

j) Within 12-18 h, bacterial growths were centrifuged (10 min at 5,000 × g) and all remnants of supernatant were removed.

k) Pellets were used for the extraction of DNA.

II.) Serial Passage:

a) Prior to beginning serial passage, steps a-m of MIC protocol were followed and initial MIC is annotated.

b) Using a 50 μL tip, the inoculum from the well just one dilution below the MIC of the previous passage (step a), was acquired by dipping tip into inoculum

c) All fluid is expelled and tip is washed in 1 mL of fresh trypticase soy broth.

d) This 1 mL of inoculated broth is used to start a new MIC, adding 90 μL into the first well of the next column available and 50 μL to wells 2 through 12.

e) Steps f-m were followed as described under MIC protocol.

f) Steps b-e were then followed for a series of 19 more MIC readings.

g) After 20 passages, purity plates are streaked from the well just one dilution below the last MIC recorded.

h) After 24 h, if mixed colony morphotypes are present, individual colonies are re-streaked onto fresh blood agar plates.

i) Colonies were picked and individually inoculated into 3 mL of TSB and grown in a 37 °C incubator at 200 rpm overnight.

j) Within 12-18 h, bacterial growths were centrifuged (10 min at 5,000 × g) and all remnants of supernatant were removed.

k) Pellets were used for the extraction of DNA.

III.) DNA Extraction (Genomic DNA Purification Kit; Thermo Scientific):

a) 400 µL of lysis solution was added to bacterial pellet and incubated at 65 °C for 5 min; or 65 °C for 10 min if samples were frozen.

b) 600 µL of chloroform was immediately added followed with gentle inversion of the sample 3-5 times.

c) Sample was centrifuged for 2 min at 10,000 rpm.

d) Precipitation solution was prepared by mixing 720 µL of sterile deionized water with 80 µL of supplied 10X concentrated Precipitation solution.

e) After centrifugation (step c), the upper aqueous phase was transferred into a new tube and 800 µL of freshly made precipitation solution (step d) was added.

f) Solution was mixed by several gentle inversions of the tub at room temperature.

g) Sample was centrifuged for 2 min at 10,000 rpm.

h) All remnants of supernatant were removed and DNA pellet was dissolved in 100 µL of 1.2 M NaCl with gentle vortexing.

i) 300 µL of cold ethanol was added for precipitation of DNA at -20 °C overnight.

j) Sample was centrifuged for 5 min at 10,000 rpm.

k) All remnants of ethanol were removed.

l) DNA pellet was dissolved in 20 µL of sterile deionized water with gentle vortexing.

m) DNA concentration was measured using the NanoDrop 1000 spectrophotometer (Version3.6.0, Thermo Scientific).

IV.) Gene Amplification (PCR):

a) Genomic DNA if needed was diluted to 100 ng/μL.

b) 1 μL of DNA was mixed with 2 μL each of forward and reverse primers (Integrated DNA Technologies; Coralville, IA) and 20 μL of Master mix (5 PRIME) and 25 μL of water was added and set into the Bio-Rad MJ Mini Thermo Cycler for gene amplification.

c) Sample concentration was measured using NanoDrop 1000 spectrophotometer (Version3.6.0, Thermo Scientific).

V.) Characterization of Mutants:

a) PCR samples (100ng/μL) along with 3 sequencing primers (R346, R792, and F712) at 10 μM were sequenced by Functional Bioscience, Inc

b) Sequence analyses were performed using Vector NTI in comparison with *P. aeruginosa* PheRS alpha subunit.

c) Histograms were looked at to determine true mutations depicted from amino acid sequence.

VI.) Mutational Modeling *E. coli* PheRS

a) Modeling was possible through the use of PyMOL software.

b) P. *aeruginosa* PheRS alpha subunit protein was aligned with *E. coli* PheRS alpha subunit protein to aid in the visual representation of mutated genomic DNA sequence in a 3D structure.

12.) Structural Activity Relationship Development:

a) Leading compounds of this experiment was the base for identification of 16 compounds from the Ambinter Chemical Compound Database.

b) Compounds selected were used for IC_{50} and MIC testing (protocol is followed as described above).

BIOGRAPHICAL SKETCH

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