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A Screening Platform for Identification of Compounds That Inhibit Protein Synthesis in Pseudomonas Aeruginosa

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A SCREENING PLATFORM FOR IDENTIFICATION OF COMPOUNDS THAT INHIBIT PROTEIN SYNTHESIS IN *PSEUDOMONAS AERUGINOSA*

A Thesis

by

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May 2014

ABSTRACT

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Pseudomonas aeruginosa is a Gram-negative pathogenic bacteria and a common cause of nosocomial infections. This pathogen has recently garnered attention due to its pan-resistance. We have developed a poly(U) mRNA-directed aminoacylation/translation (A/T) protein synthesis system from *P. aeruginosa*. The concentration of each component of the system has been arrived at through multiple rounds of optimization. Poly-phenylalanine synthesis in the system was monitored using scintillation proximity assays (SPA). This system has been used to screen two natural compound libraries (>1100) and a number of the compounds that inhibit greater than 50% of the activity of the system have been identified. The molecular targets for the hit compounds were determined. Minimum inhibitory concentrations (MIC) were determined per NCCLS guidelines. Three of the compounds were tested in time-kill assays to determine their mode of inhibition and tested for their ability to inhibit eukaryotic cytosolic and mitochondrial protein synthesis and none was determined.

DEDICATION

Dedicated to my family, especially to my husband Jiantao Zhang, my son Edward Zhang, and my daughter Maggie Angela Zhang, for their love, inspiration and support throughout the years.

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CHAPTER I

INTRODUCTION

Protein synthesis is the process by which amino acids are linearly arranged into proteins through the involvement of ribosomes, transfer RNA (tRNA), messenger RNA (mRNA), and various enzymes. In the biological system, protein synthesis is also called translation, it is part of the process of gene expression. The central dogma of genetics states that deoxyribonucleic acid (DNA) encodes ribonucleic acid (RNA) through the process of transcription, which in turn expresses proteins through the process of translation (1).

The translation process has three major steps: initiation, elongation and termination. In the initiation step, initiation factors 1, 2, 3 (IF1, IF2, IF3) function in the formation of the initiation complex. This complex contains the functional complete 70S ribosome, the mRNA attached to the ribosome with the start codon positioned on the P site, and the initiator tRNA bound at the P site by codon-anticodon pairing with the mRNA; in the elongation step, one amino acid is incorporated into the peptide with each cycle. This process requires the involvement of three elongation factors: EF-Tu, EF-Ts and EF-G; in the termination step, a stop codon will arrive at the A site and is recognized by one of the ribosome release factor 1, 2 or 3 (RRF1, RRF2, RRF3), then the newly formed peptide will be released from the ribosome. This step also requires the involvement of EF-G (2).

The protein synthesis system in this project is different from the one in the biological system because it only contains the elongation step, and the elongation step requires the aminoacylation of tRNA, therefore it is also called an Aminoacylation/Translation (A/T) system. This system contains both enzymatic and non-enzymatic components. The enzymatic components include: the ribosome, which is the universal cellular organelle that translates the genetic code by catalyzing the sequential polymerization of amino acids into proteins (3); amino acyl-tRNA synthetases (aaRS), which catalyze the attachment of an amino acid to the cognate

tRNA, this process is called aminoacylation or tRNA charging (4); elongation factor Tu (EF-Tu), delivers the charged tRNA to the A site of the ribosome in a ternary complex with a guanosine-5'-triphosphate (GTP) and an aminoacylated tRNA, hydrolyzing the GTP to guanosine diphosphate (GDP) during this process (5, 6); elongation factor Ts (EF-Ts), which serves as the guanine nucleotide exchange factor for EF-Tu, catalyzing the release of GDP from EF-Tu, enabling EF-Tu to bind to a new GTP molecule, thus regenerate the active form of EF-Tu (7); and elongation factor G (EF-G), which catalyzes the translocation of the tRNA and mRNA across the ribosome at the end of each round of polypeptide elongation (8, 9, 10). Here, phenylalaninyl tRNA synthetase (PheRS) was used as the aaRS, because the genetic code for phenylalanine is UUU and poly(U) mRNA is used as the mRNA.

The non-enzymatic components of this system includes the following molecules. tRNA, which serves as the physical link between the nucleotide sequence encoding the protein and the amino acid sequence of the protein (2). Adenosine triphosphate (ATP) and GTP, which provide energy for the protein synthesis system. Phosphoenolpyruvate (PEP) and pyruvate kinase (PK) form an energy source regeneration system to maintain constant levels of ATP and GTP (11). This energy source regeneration system is only needed in the protein synthesis system in this project, but in the biological system, it is not necessary. Mg^{2+} functions as a cofactor in a various steps in the protein synthesis system, and K^{+} is needed to stabilize several of the proteins. Dithiothreitol (DTT) is used to stabilize enzymes in the system by creating a reducing environment and preventing disulfide bond formation. Phenylalanine is the substrate, which will form poly(Phe) during the protein synthesis, and it is [^{3}H] labeled so the activity of the system can be monitored by detecting the radioactive signal. Poly(U) mRNA provides genetic code for the protein synthesis system.

Protein synthesis is a well-established target for antibiotic discovery (12, 13, 14, 15). Each of the enzymatic components: ribosome, PheRS, EF-Tu, EF-Ts and EF-G offers attractive targeting site for antibiotics. Table 1 listed the validated antibiotics that inhibit protein synthesis system in bacteria.

Table 1. Antibiotics that target the protein synthesis system in bacteria.

Antibiotics	Mode of action
● Aminoglycosides Streptomycin Kanomycin Neomycin Gentamycin	Prevents formation of initiation complex and inhibits correct decoding of mRNA.
● Tetracyclines	Prevents transfer of activated amino acid to the ribosome.
● Macrolides Erythromycin Azithromycin (Zithromax) Clarithromycin (Bioxin)	Binds 23S RNA and blocks elongation of nascent peptide.
● Lincosamides Clindamycin (Cleocin)	Binds 23 S RNA and blocks elongation of nascent peptide.
● Streptogramins Quinupristin (Symercid) Dalfopristin	Binds 23 S RNA and blocks elongation of nascent peptide.
● Aminocyclitols Spectinomycin	Interferes with 30 S ribosomal subunit activity.
● Oxazolidinones Linezolid	Interferes with initiation complex formation.
● Fusidic Acid Fusidin Leo	Prevents EF-G dissociation.
● Kirromycin	Binds EF-Tu and prevents its interaction with tRNA/EF-Ts.
● Thiostrepton	Binds 23 S RNA and blocks EF-G interaction.
● Puromycin	Causes premature termination of peptidyl-transfer.
● Chloramphenicol	Inhibits peptidyl-transferase.

Resistance has been detected in almost all these antibiotics, and some bacteria have acquired resistance to more than one or even all of the antibiotics that are currently being used. Therefore, there is a critical need for new novel antibiotics with a different mechanism of action.

The goal of this project is to develop a screening platform based on the protein synthesis system from *P. aeruginosa* and use this system to screen for inhibitors of function to identify compounds that may be developed into new antibiotics. This includes: 1) Purification of all the protein components in large scale; 2) Development of a functional assay to monitor protein synthesis; 3) Optimization of the protein synthesis system by titration of each of the components; 4) Use of the optimized system to screen chemical compounds; 5) Determination of antibacterial activity of the inhibitor of the system.

CHAPTER II

LITERATURE REVIEW

Multidrug-resistant (MDR)

Multidrug-resistant (MDR) is defined as 'acquired non-susceptibility to at least one agent in three or more antimicrobial categories' (16) by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC). Emergence of resistance to 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. First, there are fewer, or sometimes even no effective antimicrobial agents available for treatment of infections caused by these bacteria (17, 18). Second, the fact that antibiotic-resistant infections have been noted to double during the hospital stay. Third, antibiotic-resistant infections caused double mortality and probably double morbidity (and presumably the costs) as compared with drug-susceptible infections (19). Furthermore, if a bacterium is resistant to one antibiotic it is more likely to obtain resistance to other antibiotics than an isolate of the same species which is susceptible to that particular antibiotic (20).

Fundamental mechanisms of MDR

Bacteria can acquire antibiotic resistance through different means. The fundamental mechanisms including reduced permeability or uptake, enhanced efflux, enzymatic inactivation and alteration or over-expression of the drug target (21).

Typical multidrug resistant bacteria

The multidrug resistant pathogens encompasses both Gram-positive and Gram-negative organisms, and there are four typical multidrug resistant bacteria. These so-called 'superbugs' include methicillin and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) (22), macrolide-resistant *Streptococcus pneumoniae* (23), vancomycin-resistant *Enterococci* (24) and pan-resistant *Pseudomonas aeruginosa* (25). Of these pathogens, *P. aeruginosa* is the focus of our research, so it will be described in greater detail.

P. aeruginosa is an aerobic Gram-negative bacterium that is an important cause of both community-acquired and hospital-acquired (nosocomial) infections. *P. aeruginosa* is one of the leading nosocomial pathogens and it is responsible for 10-15% of nosocomial infections worldwide (26). Nosocomial infections caused by *P. aeruginosa* include pneumonias, urinary tract infections (UTIs), bloodstream infections, surgical site infections and skin infections in the setting of burn injuries. Chronic sinopulmonary colonisation and recurrent infections from *P. aeruginosa* are seen in patients with cystic fibrosis (CF). Community-acquired infections include, but are not limited to, ulcerative keratitis (usually associated with contact lens use), otitis extema (typically in immunocompromised hosts such as those with diabetes mellitus), and skin and soft tissue infections (including diabetic foot infections) (27, 28).

Infections caused by *P. aeruginosa* are not only common, but they have also been associated with high morbidity and mortality when compared with other bacterial pathogens. *P. aeruginosa* has recently garnered the most attention due to its pan-resistance to most antibiotic agents.

Nosocomial infections caused by *P. aeruginosa* are usually difficult to treat because of both the intrinsic resistance of the species (it has constitutive expression of Amp C β-lactamase and efflux pumps, combined with a low permeability of the outer membrane) (29), and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents (30), including β-lactams, aminoglycosides and fluoroquinolones.

What makes *P. aeruginosa* clinically so important is that practically all known mechanisms of antimicrobial resistance has been detected in this organism. These mechanisms include: derepression of chromosomal Amp C cephalosporinase; production of plasmid or integrin mediated β-lactamases from different molecular classes (carbenicillinases and extended spectrum β-lactamases belonging to class A, class D oxacillinases and class B carbapenem hydrolyzing enzymes); diminished outer membrane permeability (loss of Opr D proteins); overexpression of active efflux systems with wide substrate profiles; synthesis of aminoglycoside modifying enzymes (phosphoryltransferases, acetyltransferases and adenylyltransferases); and structural alterations of topoisomerases II and IV determining quinolone resistance (31).

As described above, antibiotic resistant is a serious problem worldwide, however, the antibiotics available each year has not increased accordingly. Alternatively, new antibiotics

approved by FDA have decreased dramatically every 5 years since 1983 (32) (Fig. 1). To add to this, many of the new antibiotics are only modified forms of the previously marketed antibiotics. While the derivatization of existing antibiotics by pharmaceutical companies has so far been successful, this strategy is short-term, and the discovery of antibiotics with novel scaffolds would be a greater contribution in the fight against multidrug-resistant infections. Therefore, there is a critical need for new novel antibiotics with a different mechanism of action.

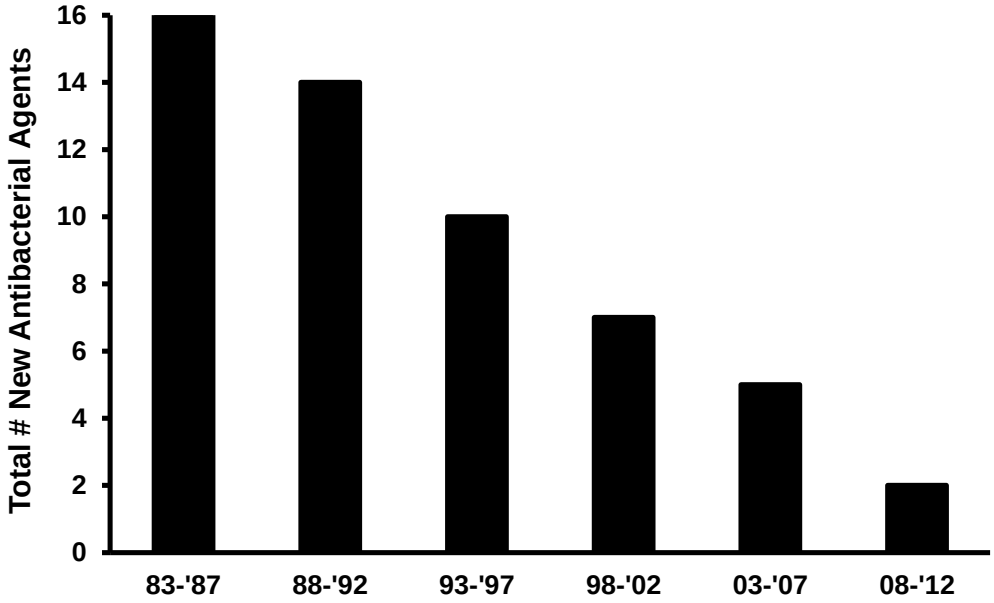


Fig 1. New Molecular Entity (NME) Systemic Antibiotics Approved by the US FDA per Five-year Period (32).

Screening strategies

Different strategies have been used for identifying novel antibacterial compounds, and each of these methods has its pros and cons. These methods include whole cell screening, cell extracts screening and target based screening.

A traditional way of proceeding is whole cell screening. This involves microbiological primary screenings to identify hits having antimicrobial activity against growing cultures. And followed by secondary screenings which is aimed at obtaining a higher level of characterization of the hits and at the possible selection of lead compounds. (33). This type of approach had some success in the past, but it is regarded as outdated today since it would likely 'rediscover' known inhibitors and would also miss a large number of potentially interesting molecules that may not penetrate the test cells (34, 35). Also, a major consideration in the use of whole cell screening is that large differences in sensitivity may exist among different bacteria (36).

In general, Gram-negative bacteria have been observed to be more difficult targets for antibiotics than Gram-positive bacteria because the outer membrane of Gram-negative bacteria is more difficult to penetrate and because multiple types of antibiotics are expelled by the efflux pumps, which are major non-plasmid-encoded contributors to multidrug resistance in Gram-negative bacteria. However, different sensitivity to antibiotics is not restricted to differences between Gram-positive and Gram-negative bacteria, but is also observed between different species of the same genus or among different strains of the same species, which have substantial variations in their genome. It is therefore advisable to screen against clinical isolates of interest rather than against model laboratory strains (37, 38). Thus, improved methods of whole cell screening are necessary for success. Another problem with whole cell screening is the use of clinically pathogenic bacteria, thus increasing safety concerns.

Attempts have also been made to screen chemical compound libraries by using cell extracts containing native transcription and translation systems from *Escherichia coli* (39), *Streptococcus pneumoniae* (40, 41), *Staphylococcus aureus* (42) and *Pseudomonas aeruginosa* (43). However, this approach has had only limited success. The use of cell extracts for screening can be problematic due to the presence of nucleases, degraded nucleic acids, soluble but denatured proteins, and turbidity (44). In addition, different preparations of S30 fractions can differ in activity and are therefore undependable (45).

Another approach is target based screening, which is also the strategy used in this project. This approach offers the advantage of identifying inhibitors having the desired biological activity. Approaches of this type have failed in a number of cases because of the failure of the inhibitors to penetrate and remain active in bacterial cells. However this is a valid strategy and with the use of knowledge-based modifications could yield potential lead compounds (46). The problem of cell permeability may be bypassed by subsequent chemical modifications, e.g. by rendering the inhibitor more hydrophobic or linking it to a cell-permeable molecule such as a polyamine, a sugar or to another validated antibiotic (47, 48).

Scintillation proximity assay (SPA) technology

Scintillation proximity assay (SPA) technology was used to detect compounds that inhibit activity during this project. In SPA, the target of interest is immobilized by binding to a small scintillant containing microsphere, approximately 5 pico-meter (pm) in diameter. When a radioisotopically labelled molecule binds to the microsphere, the radioisotope is brought into

close proximity to the scintillant and effective energy transfer from the emitted β-particle will take place, resulting in the emission of light. The radioisotopes remaining free in solution will be too distant from the scintillant, and the β-particle will therefore dissipate the energy into the aqueous medium and remain undetected (49, 50, 51, 52).

CHAPTER III

MATERIALS AND METHODS

Gel Electrophoresis and Protein Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4 to12% (w/v) polyacrylamide precast gels (Biorad). Benchmark unstained protein molecular weight markers were from Invitrogen (Madison, WI). Protein concentrations were determined by the method of Bradford (53) using Coomassie Protein Assay Reagents (Thermo Scientific) and bovine serum albumin as the standard.

Purification of proteins

Cultures were grown in Terrific Broth containing 25 μg/ml of kanamycin and 50 μg/ml of chloramphenicol for *P. aeruginosa* PheRS, EF-G (54) and EF-Ts (55) or 50 μg/ml of ampicillin and 50 μg/ml of chloramphenicol for *P. aeruginosa* EF-Tu (55) or 25 μg/ml of ampicillin and 25 μg/ml of kanamycin for human mitochondrial PheRS (hmPheRS) (56) at 37 °C. Expression of the target proteins were induced at an optical density (A_{600}) of 0.6-0.8 by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to 0. 5 mM for *P. aeruginosa* PheRS, EF-G and EF-Ts, 0.25 mM for *P. aeruginosa* EF-Tu and 50 μM for hmPheRS. Growth of the bacterial culture was continued for 3 h post induction and the bacteria were harvested by centrifugation (10000 × g, 30 min, 4 °C). The cells were lysed and Fraction I lysate was prepared as previously described (57).

P. aeruginosa PheRS and EF-G were precipitated between 35 and 55% saturation of ammonium sulfate (AS), *P. aeruginosa* EF-Tu was precipitated between 45 and 60% saturation of ammonium sulfate, *P. aeruginosa* EF-Ts was precipitated between 0 and 55% saturation of ammonium sulfate and hmPheRS was precipitated between 0 and 60% saturation of ammonium sulfate. The precipitated proteins were collected by centrifugation (23,000 × *g*, 60 min, 4 °C). All proteins were further purified to more than 98% homogeneity using nickel-nitrilotriacetic acid (NTA) affinity chromatography (Perfect Pro, 5 Prime) followed by dialysis (two times)

against a buffer containing: 20 mM Hepes-KOH (pH 7.0), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10 % (v/v) glycerol. Purified proteins were fast frozen in liquid nitrogen and stored at -80 ºC.

Aminoacylation/translation assays

A scintillation proximity assay (SPA) was developed for the aminoacylation/translation (A/T) assay. The complete assay contained 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 10 mM $MgCl_2$, 0.03 mM spermine, 1.5 mM ATP, 0.5 mM GTP, 40 µM [^{3}H]Phenylalanine (75 cpm/pmol) and 0.3 mg/ml poly(U). To maintain constant levels of ATP and GTP the assay contained a nucleotide regeneration system composed of 4 mM phosphoenolpyruvate (PEP) and 0.025 Units/µl pyruvate kinase (PK). The concentration of *P. aeruginosa* ribosomes and proteins in the assay were as follows: ribosome (0.2 µM), PheRS (0.1 µM), EF-Tu (1 µM), EF-Ts (0.05 µM) and EF-G (0.2 µM). These concentrations were arrived at through sequential rounds of optimization: each concentration represents the concentration just below the saturation point of the titration.

The screening reactions were carried out in 96-well microtiter plates (Costar). Test compounds were equilibrated by addition of 33 µl of the protein/substrate mix (without tRNA) to 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 then reactions were initiated by addition of 15 µl of *E. coli* tRNA (20 µM), followed by a 2 hour incubation at room temperature (comparable to 1 hour at 37 °C). Reactions were stopped by the addition of 5 µl of 0.5 M 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 a1450 Microbeta Jet Liquid Scinitllation and Luminescence Counter (Wallac).

Assays to confirm the primary hits were as described above except that only primary hit compounds identified from the initial screening were used to identify false negatives. These assays were carried out in duplicate to reliably confirm results.

Assays to determine IC_{50} values were as described above with the test compounds serially diluted from 200 µM to 0.4 µM. The concentration ranges of antibiotic in control plates were as follows: spiramycin (0.02 µM to 20.0 µM), tylosin (0.02 µM to 20.0 µM), fusidic acid (4 µM to 512 µM).

PheRS assay

SPA assays to determine inhibition of PheRS by chemical compounds were as described (58) with the exception that the enzyme mix was pre-incubated with 132 µM chemical compound for 15 min prior to addition of tRNA. The 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 the plates were analyzed as above. Assays to determine IC_{50} values of the inhibitors that inhibit PheRS were as described above with the test compounds serially diluted from 200 µM to 0.4 µM.

Assays to determine inhibition of hmPheRS by chemical compounds were as described (56) with the exception that the enzyme mix was pre-incubated with 0.4 µM to 200 µM chemical compounds for 15 min prior to addition of tRNA.

EF-Tu and EF-Ts GDP exchange assay

Nitrocellulose binding assays were used to determine inhibition of GDP exchange by EF-Tu as previously described (59) with the exception that the enzyme (1.0 µM) was pre-incubated with 132 µM chemical compound for 15 min prior to the addition of [^{3}H]GDP. EF-Ts stimulates exchange of GDP bound by EF-Tu. The ability of compounds to inhibit EF-Ts stimulation of GDP exchange by EF-Tu was measured in assays as described for EF-Tu/GDP exchange with the exceptions that EF-Ts was present (0.05 µM), and the time for the reaction was decreased from 30 min to 30 sec.

EF-G GTPase assay

Assays to determine inhibition of ribosome-dependent GTPase activity of EF-G were carried out in 50 µl reactions containing: 50 mM Tris-HCl, (pH 7.5), 10 mM $MgCl_2$, 70 mM NH_4Cl, 1 mM dithiothreitol (DTT), 1.8 mM GTP, 0.2 µM EF-G, 0.4 µM *P. aeruginosa* ribosomes and 132 µM test compounds. Assays were stopped by the addition of 150 µl of 50 mM ethylenediaminetetraacetic acid (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) per manufacturer's directions. Assays to determine IC_{50} values of the inhibitors that inhibit the GTPase activity of EF-G were as described above with the test compounds serially diluted from 200 µM to 0.4 µM.

Minimum Inhibitory Concentration (MIC) testing

Broth microdilution MIC testing was performed in 96-well microtiter plates according to National Committee for Clinical Laboratory Standards (NCCLS) document M7-A6 (60). MIC values were determined against 10 pathogenic bacteria: *Moraxella catarrhalis* (ATCC 25238), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pneumonia* (ATCC 49619), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Haemophilus influenza* (49766), *Pseudomonas aeruginosa* (ATCC 47085). All bacterial strains were acquired from the American Type Culture Collection (Manassas, VA). *P seudomonas aeruginosa Hypersensitive strain* (ATCC 35151), *P seudomonas aeruginosa PA200 Efflux* (efflux pump mutant), *E. coli TolC Efflux* (efflux pump mutant) were a kind gift from Urs Ochsner (Crestone Pharma-Boulder CO). The inhibitors were serially diluted from 128 µg/ml to 0.125 µg/ml in 96-well plates (costar) and incubated with the bacteria until the OD_{625} value reached above 0.2 and were then diluted to 0.08 to 0.13. This concentration is equivalent to $0.5\text{-}1\times10^{8}$ CFU (colony forming units). Incubation was continued for 18-24 h and the MIC was reported as the lowest concentration of inhibitor that prevent visible bacterial growth.

Time kill study

Time-kill study was performed using *M. catarrhalis* and *E. faecalis* according to the NCCLS document M26-A (61). Growth media were Brain Heart Infusion and Trypticase Soy Broth from Remel (Lenexa, KS). For the experiments, 10 ml of broth medium was inoculated with 0.1 ml of a fresh overnight growth culture and grown at 35 ºC with shaking (200 rpm) for 2-3 hours. Pre-warmed flasks containing 10 ml of medium alone or 10 ml of medium with compound (BM_03E08, BM_04E04 or BM_07F03) at 4 × MIC were then inoculated with 0.1 ml of the exponentially growing cultures which was diluted to OD_{625} in the range of 0.08 to 0.13.Samples were removed at 0, 2, 4, 6, and 24 h, and serial dilutions were plated on blood agar to allow for colony enumeration and calculation of the live cell density the following day.

Eukaryotic protein synthesis assay

Reactions contained 60% wheat germ extract (Promega), 3.0 µg poly(U) mRNA, 75.0 µM [^{3}H]Phenylalanine (75 cpm/pmol), 2.0 µM yeast $tRNA^{Phe}$ and 6.0 mM MgOAc. All components were assembled on ice, mixed with compound and incubated at ambient temperature for 2 hours. Reactions were stopped by addition of 2 ml of 5% trichloroacetic acid (TCA), heated to 90 °C

for 15 min and filtered through glass-fiber filters (Whatman). Concentrations of compounds ranged from 0.8 to 200 μM.

Binding mode assay

To determine competition with ATP, IC50s were determined in SPA reactions containing varying ATP concentrations. A mix (33 μl) containing 50 mM Tris-HCl (pH 7.5), 0.5 mM spermine, 8 mM MgCl2, 100 μM [3H]Phe (75 cpm/pmol), 1 mM DTT, indicated concentrations of ATP (25, 50, 100, 250, 500, 1000 μM), and 0.05 μM P. aeruginosa PheRS was added to the compound (2 μl). Final compound concentrations in the reactions ranged from 200 to 0.4 μM. The mix was allowed to incubate at room temperature for 15 min. The reaction was started by addition of 15 μl tRNA (80 μM tRNA or 2 μM tRNA-Phe). Positive controls contained only DMSO without compound. The reactions were for 1 hour at 37 ºC and stopped by the addition of 5 μl of 0.5 M EDTA.

To determine competition with Phe the same assay was used. However, ATP was held at a constant concentration of 2.5 mM in assays containing indicated concentrations of Phe (25, 50, 100, 200, 300 μM). Background amounts of free [3H]Phe in the absence of PheRS were insignificant.

CHAPTER IV

RESULTS

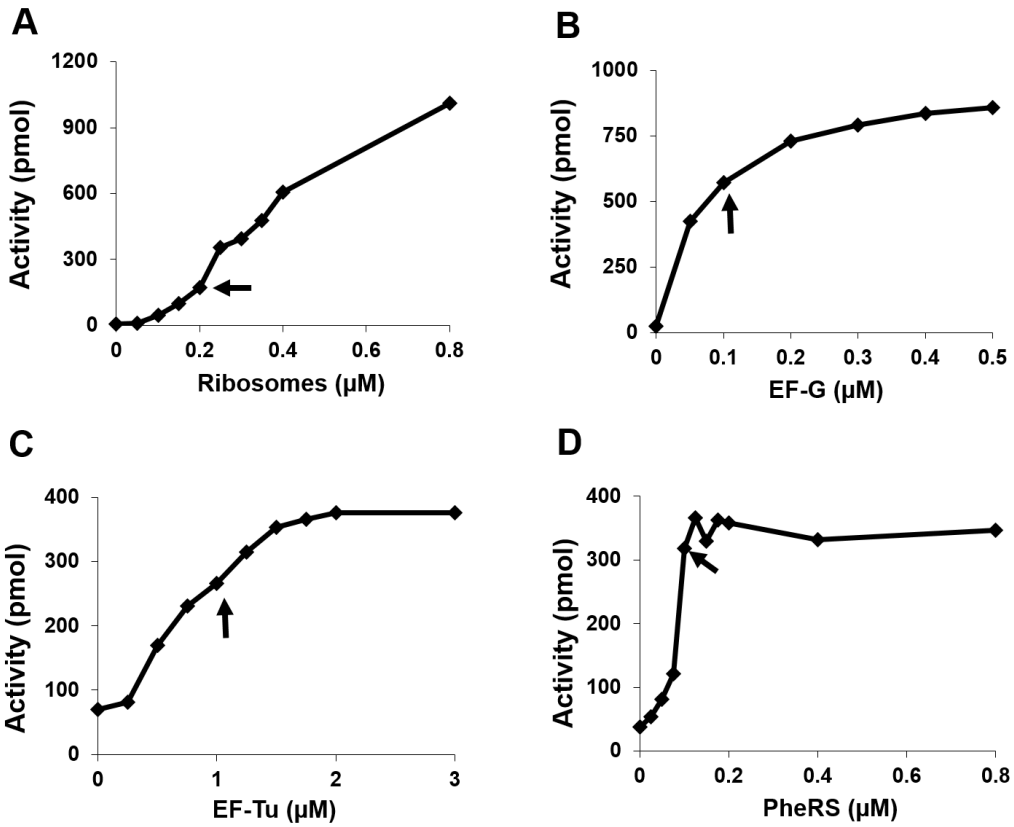
Development and Optimization of the A/T Assay

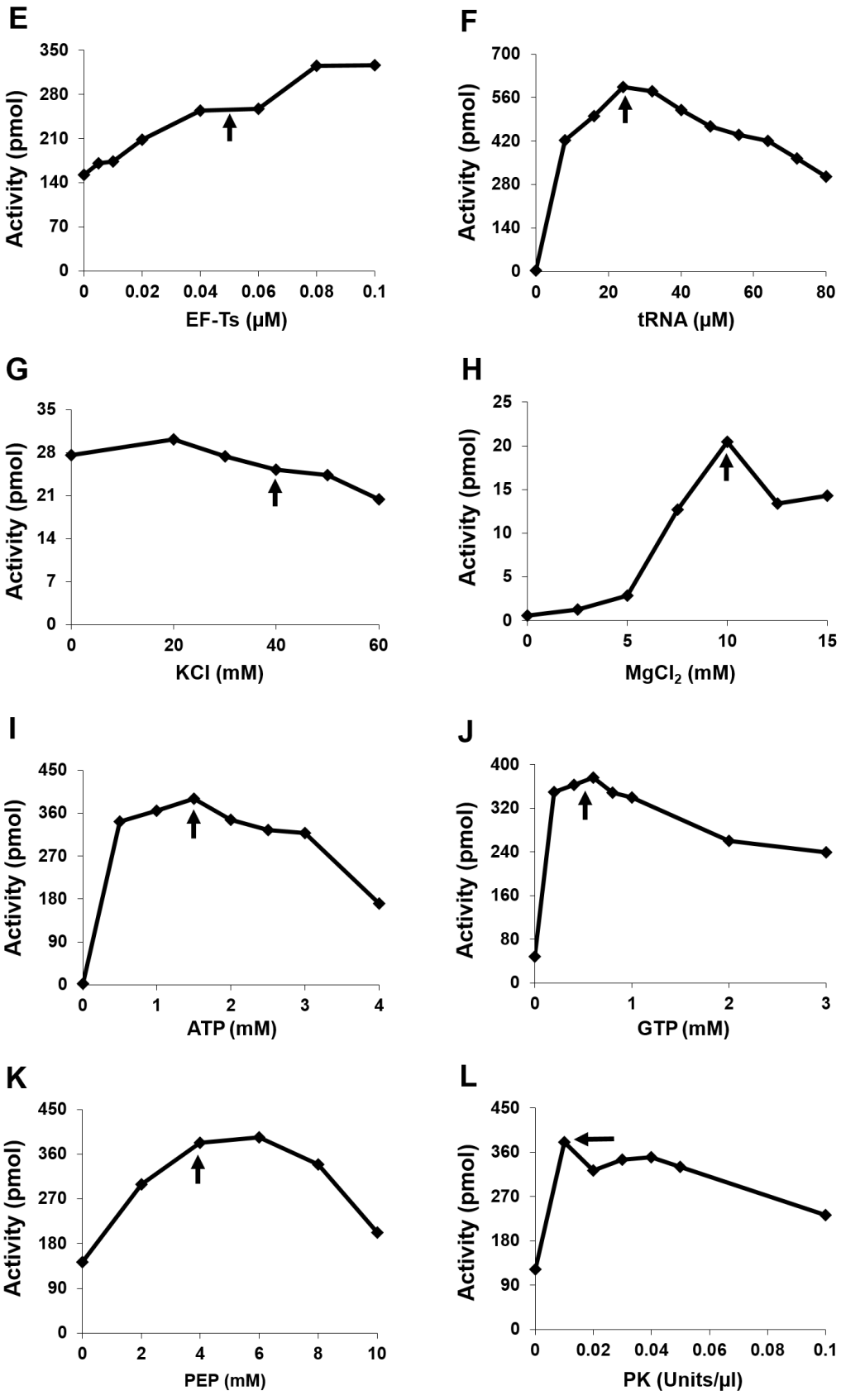
Titration of each component in the A/T assay

An aminoacylation/translation (A/T) system was developed that contained all the components from *P. aeruginosa* required for translation of poly(U) mRNA. The components included ribosomes, EF-Tu, EF-Ts, EF-G, PheRS and Poly(U) mRNA, ATP, GTP, PEP, PK, Mg^{2+}, K^{+}, spermine, DTT, pH 7.5 Tris-Cl buffer, H_2O and tRNA. The assay was optimized for screening in a 96 well micro-titer plate format.

Ribosomes were titrated first while keeping all the other components at saturated concentrations. The concentration of ribosomes to be used in the screening assay was determined from the titration. Components of the assay were titrated individually into the assay. The concentration of each components giving optimal conditions for screening was determined. When the appropriate concentration for one component was determined, that concentration was used while optimizing other components. At the conclusion of one round of the titration, new concentrations for each of the components were determined. The new concentrations were used in following rounds of titration. When the concentration of one component was changed, all the other components were re-titrated. After sequential rounds of titration, the optimal concentration for each component was arrived at (Table 2).

The titration curves for each component is shown in Fig 2. The arrows indicate the optimized concentrations. For the enzymatic components (ribosome, EF-G, EF-Tu, EF-Ts and PheRS), the concentration was set just below the inflection point of saturation on the titration curve to facilitate maximum sensitivity to inhibition. For the non-enzymatic components (Poly(U) mRNA, ATP, GTP, PEP, PK, Mg^{2+}, K^{+}, spermine, DTT and tRNA), the final concentration was set above the saturation point on the titration curve to avoid limitation.


A
Activity (pmol)
1200
900
600
300
0
0
0.2
0.4
0.6
0.8
Ribosomes (μM)
B
Activity (pmol)
1000
750
500
250
0
0
0.1
0.2
0.3
0.4
0.5
EF-G (μM)
C
Activity (pmol)
400
300
200
100
0
0
1
2
3
EF-Tu (μM)
D
Activity (pmol)
400
300
200
100
0
0
0.2
0.4
0.6
0.8
PheRS (μM)


E
Activity (pmol)
350
280
210
140
70
0
0
0.02
0.04
0.06
0.08
0.1
EF-Ts (μM)
F
Activity (pmol)
700
560
420
280
140
0
0
20
40
60
80
tRNA (μM)
G
Activity (pmol)
35
28
21
14
7
0
0
20
40
60
KCl (mM)
H
Activity (pmol)
25
20
15
10
5
0
0
5
10
15
MgCl2 (mM)
I
Activity (pmol)
450
360
270
180
90
0
0
1
2
3
4
ATP (mM)
J
Activity (pmol)
400
320
240
160
80
0
0
1
2
3
GTP (mM)
K
Activity (pmol)
450
360
270
180
90
0
0
2
4
6
8
10
PEP (mM)
L
Activity (pmol)
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360
270
180
90
0
0
0.02
0.04
0.06
0.08
0.1
PK (Units/μl)

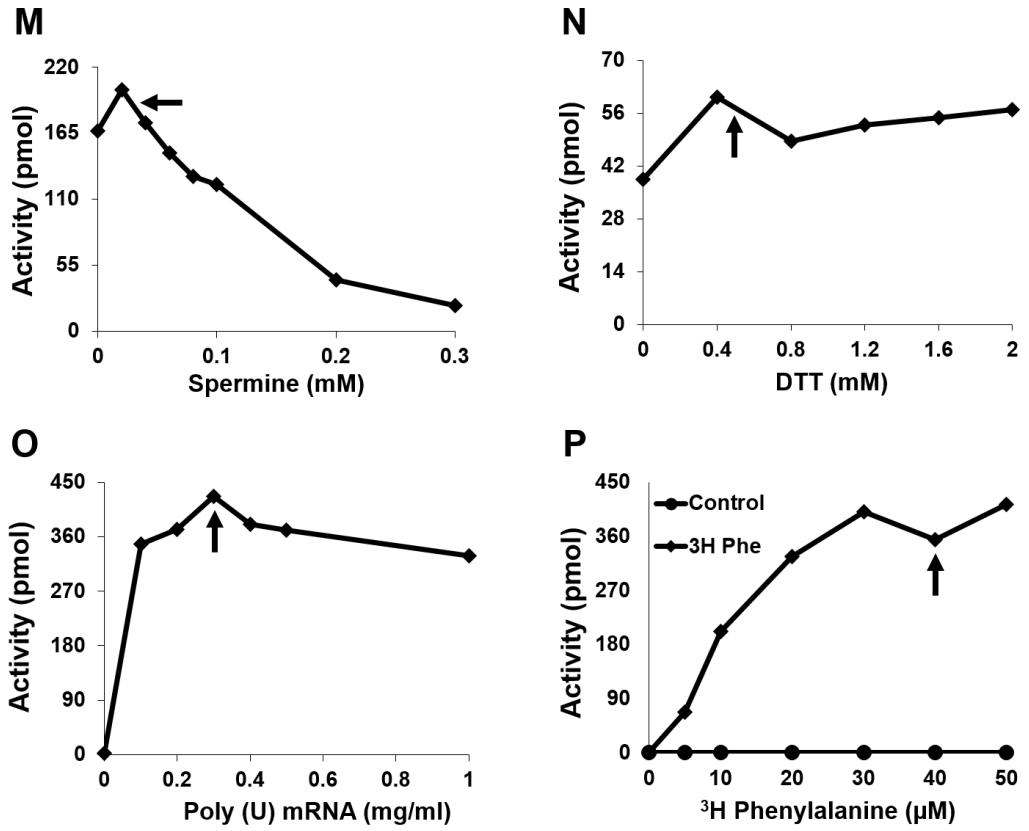


Fig 2. Optimization of the coupled aminoacylation/translation (A/T) assay in *P. aeruginosa*. (A) Titration of *P. aeruginosa* ribosomes in the A/T protein synthesis system assay. Ribosomes (0.05 to 0.8 µM) were assayed in the presence of saturating amounts of the other components. (B) Titration of *P. aeruginosa* EF-G (0.05 to 0.5 µM). (C) Titration of *P. aeruginosa* EF-Tu (0.25 to 3 µM). (D) Titration of *P. aeruginosa* PheRS (0.025 to 0.8 µM). (E) Titration of *P. aeruginosa* EF-Ts (0.005 to 0.1 µM). (F) Titration of tRNA (8 to 80 µM). (G) Titration of KCl (20 to 60 mM). (H) Titration of $MgCl_2$ (2.5 to 15 mM). (I) Titration of ATP (0.5 to 4 mM). (J) Titration of GTP (0.2 to 3 mM). (K) Titration of PEP (2 to 10 mM). (L) Titration of PK (0.01 to 0.1 Units/µl). (M) Titration of spermine (0.02 to 0.3 mM). (N) Titration of DTT (0.4 to 2 mM). (O) Titration of poly(U) mRNA (0.1 to 1 mg/ml). (P) Titration of [^{3}H] Phenylalanine (0.25 to 3 µM). The arrows indicate the concentration of each component used in the final screening assay.

Table 2. Concentration of each component of the coupled aminoacylation/translation (A/T) system used in the screening assay.

Protein Synthesis Component	Optimized Concentration
ribosomes	0.2 µM
PheRS	0.1 µM
EF-Tu	1.0 µM
EF-G	0.2 µM
EF-Ts	0.05 µM
[^{3}H] Phenylalanine	40 µM
PolyU RNA	0.3 mg/ml
ATP	1.5 mM
GTP	0.5 mM
$MgCl_2$	10 mM
KCl	25 mM
PEP	4 mM
PK	0.025 Units/µl
Spermine	0.03 mM
DTT	1 mM
tRNA	20 µM

Comparison of A/T assay activity at 37 ºC vs room temperature

Assays to compare the A/T assay activity at 37 °C vs room temperature (RT) was conducted. Activity was measured at time intervals between 15 min to 180 min. The results are shown in Fig 3. From the results, the reaction carried out at room temperature for 2 hours was observed to be equivalent to activity at 37 °C for 1 hour. At these times the signal compared to background was sufficient to allow determination of inhibitors of the assay. The screening assays for 2 h at room temperature were used in the high throughput screening of numerous plates at the same time.

Determination of the effect of DMSO on the A/T assay

All the chemical compounds supplied are dissolved in DMSO (dimethyl sulfoxide), therefore the effect of DMSO on the A/T assay was determined (Fig 4). 1% to 10% DMSO was added to

the A/T assay. Results indicated that at DMSO concentrations of 1% to 5% there was little effect on the assay activity. However, when DMSO concentration were increased from 6% to 10% a gradual decrease in activity to less than 50% of the initial activity was observed. In the screening assay, 2 µl of chemical compound was added to 50 µl assays yielding a DMSO concentration of 4%. At this point, the DMSO effect is negligible and does not interfere with the screening results.

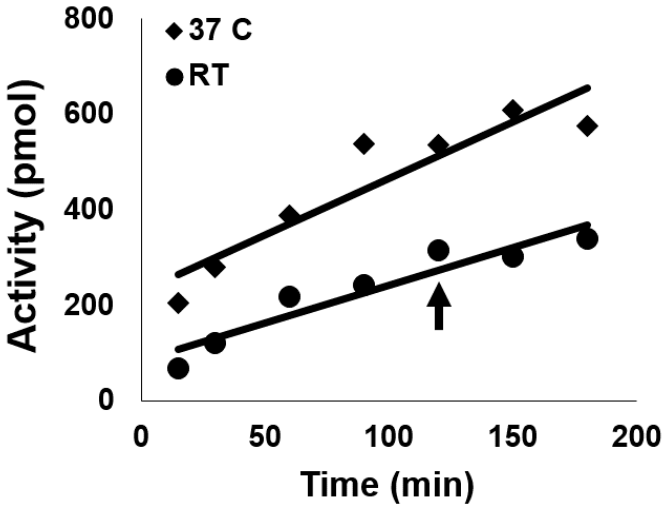


Fig 3. Comparison of protein synthesis activity at 37 ºC and room temperature (RT) at intervals between15 and 180 min. The arrow indicates the activity of the reaction at room temperature for 2 h. Filled circles (●) represents assays carried out at room temperature and diamonds (♦) represents assays carried out at 37 °C.

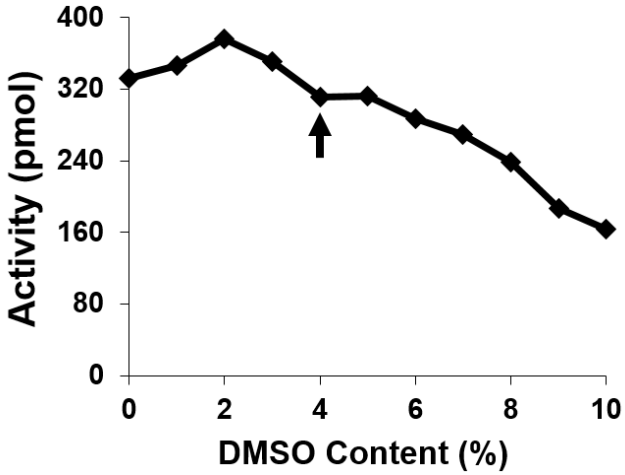


Fig 4. Detection of the effect of DMSO on the A/T assay. To achieve a compound concentration of 132 µM in the screening assay required addition of 2 µl compound to a 50 µl reaction resulting in 4% DMSO in the final assay. Arrow indicates 4% DMSO, which was used in all the screening assays.

Rationale and development of positive controls

Tylosin, spiramycin and fusidic acid were selected as control antibiotics. Spiramycin and tylosin are macrolides, they both contain a 16-member lactone ring and a disaccharide at the C5 position on the lactone ring. These two antibiotics inhibited synthesis of poly(Phe) by binding the 50S ribosomal subunit inside the nascent peptide exit tunnel near the peptidyl-transferase (PT) center thereby stopping peptide synthesis (62). Fusidic acid traps EF-G in the post-translocation site on the ribosome after hydrolysis of GTP (63) and in this state any further GTPase activity stops. All three antibiotics were effective in inhibiting poly(Phe) synthesis in the A/T assay with IC_{50} values of 0.20 µM, 0.31 µM and 10.4 µM, respectively (Fig 5).

At this point, the protein synthesis system has been successfully developed and optimized and ready for use in chemical compound screening. Next, the A/T protein synthesis system was used to screen over 1100 chemical compounds for inhibitors of function.

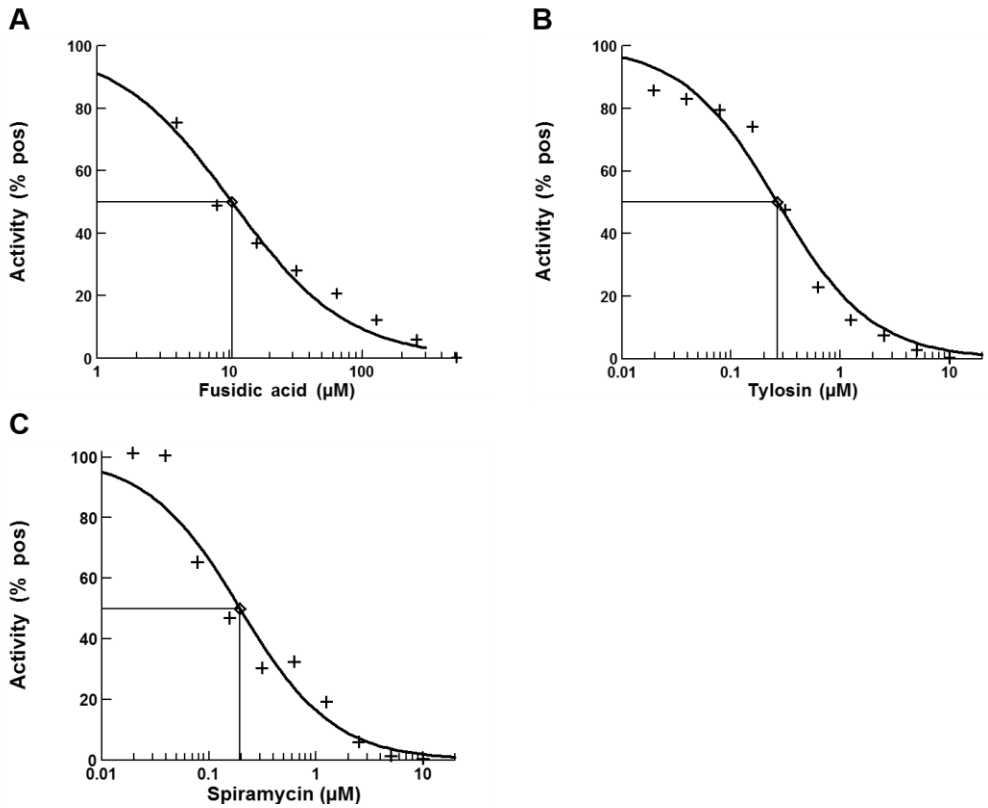


Fig 5. IC_{50} determination for positive controls in the A/T assay: (A) Fusidic acid. (B) Tylosin. (C) Spiramycin. The concentrations of tylosin, spiramycin and fusidic acid ranged from 0.02 to 20 µM, 0.02 to 20 µM and 4 to 512 µM, respectively. The antibiotics were dissolved in DMSO so that the concentration of DMSO was the same as that used in the screening assays. The curve fits and IC_{50}s were determined using XL*fit* (version 5.1; IDBS) as part of Microsoft Excel.

Screening of chemical compound libraries

Screening of chemical compound library #1

Initial screening. Using the optimized A/T system chemical compound library #1 containing 320 natural product chemical compounds was screened as described in 'materials and methods'. Positive controls contained 2 µl DMSO and were located in column 1 row A, B, C, D and column 12 row E, F, G, H in the 96-well plate. Negative controls contained 2 µl 0.5 M pH 8.0 EDTA and were located in column 1 row E, F, G, H and column 12 row A, B, C, D in the 96-well plate. The remaining 80 wells of the plate contained 2 µl of 3.3 mM chemical compound resulting in a final concentration of 132 µM.

The results were plotted using a 3-D scatter graph using JMP Pro 10.0.2 software. In the graph, the blue circles represent DMSO control, which is the positive control of the A/T assay; the orange circles represent EDTA control, which is the negative control of the A/T assay; the red circles represent primary hits, less than 50% activity of the positive control; the green circles represent all other chemical compounds in the chemical compound library #1. From the initial screening of chemical compound library #1, 19 compounds were identified, which were inhibited to less than 50% of the DMSO control (Fig 6).

Confirmation of primary hits. Duplicate assays were carried out to confirm the inhibitory activity of the 19 primary hits. Seven of the initial hits were confirmed to inhibit greater than 50% of the activity of the system (BP_01F06, BP_01C09, BP_02H03, BP_02G06, BP_02G07, BP_03B04, BP_03C05) (Fig 7). These seven compounds were carried forward in additional analysis.

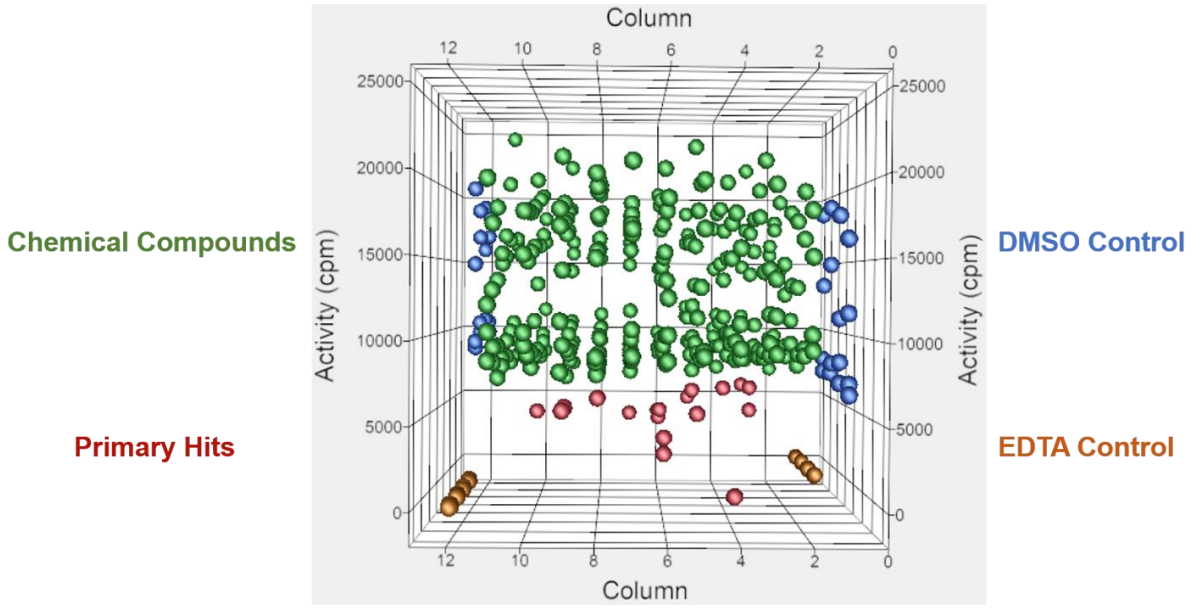


Fig 6. Initial screening results of chemical compound library #1. In the graph, the blue circles represent DMSO control, which is the positive control of the A/T assay; the orange circles represent EDTA control, which is the negative control of the A/T assay; the red circles represent our primary hits, which is below 50% activity of the positive control; the green circles represent all other chemical compounds in the chemical compound library #2.

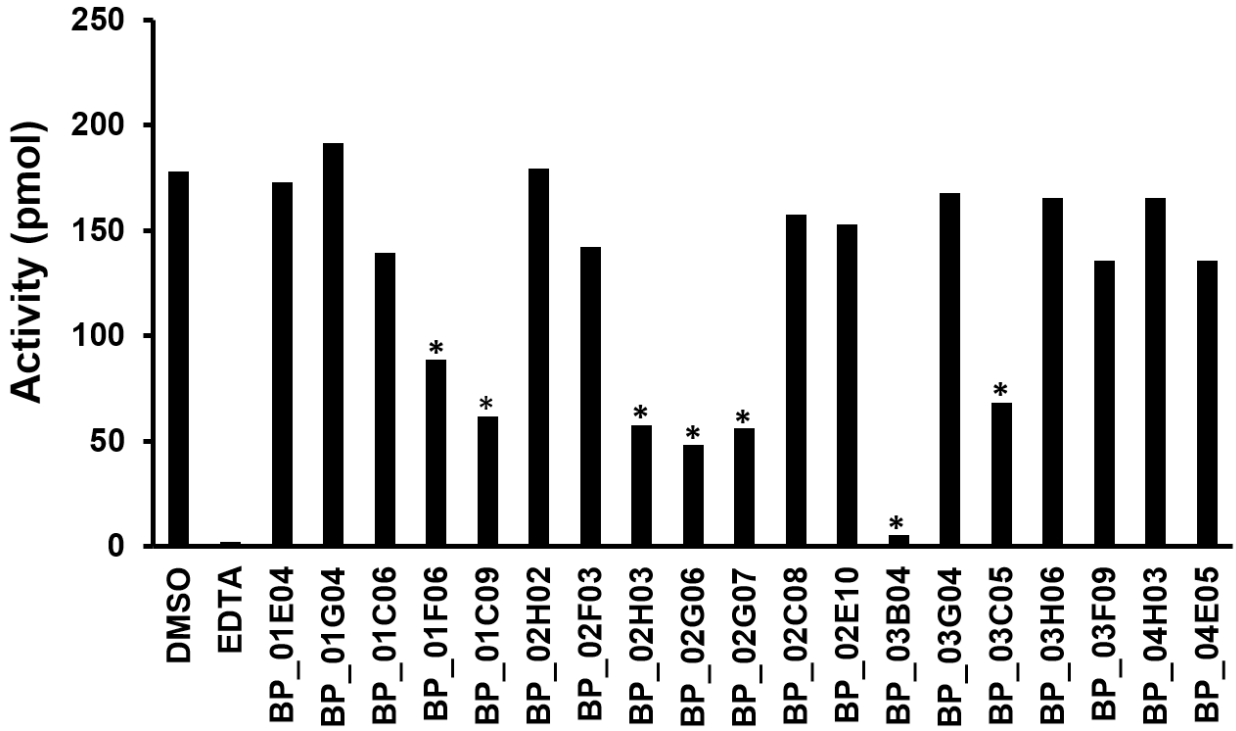


Fig 7. Confirmation of chemical compound library #1 primary hits.

IC_{50} assay of chemical compound library #1 hits. Assays to determine the concentration of inhibition to 50% of the activity of the system (IC_{50}) of the 7 confirmed hits were as described in 'materials and methods'. The chemical compounds were diluted in serial dilutions between 5 and 0.01 mM resulting in concentrations between 200 and 0.4 µM in the final assay. The reaction conditions were the same as the initial screening assay. The data were analyzed using XL fit (version 5.1; IDBS) as part of Microsoft Excel (Fig 8). The IC_{50} of the inhibitors ranged from 2.1 to 41.3 µM, with BP_02G07 giving the lowest IC_{50} (2.1 µM), and BP_02G06 giving the highest IC_{50} (41.3 µM). The IC_{50} values for chemical compound library #1 hits are shown in table 3.

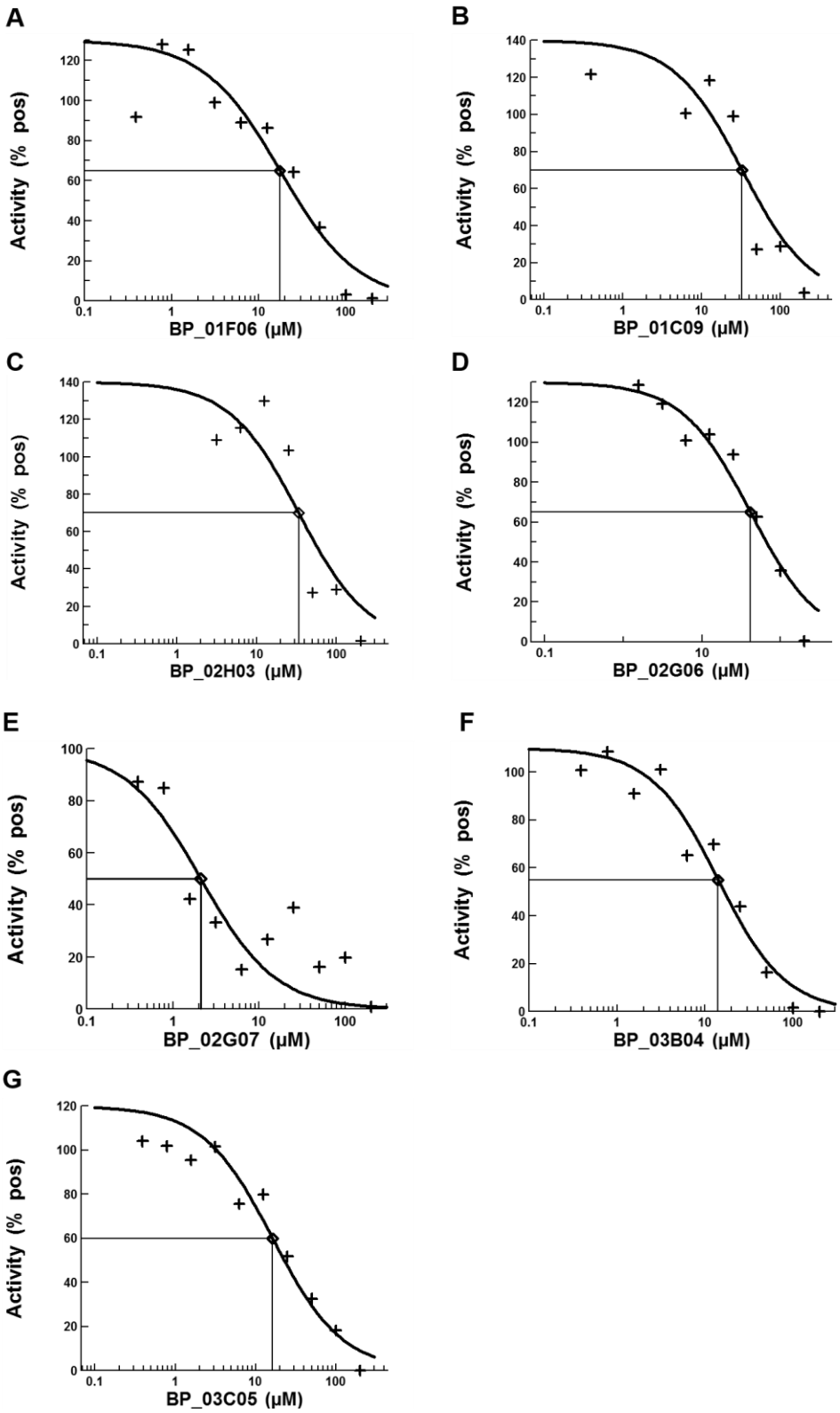


Fig 8. IC_{50} determination for chemical compound library #1 hits. (A) BP_01F06; (B) BP_01C09; (C) BP_02H03; (D) BP_02G06; (E) BP_02G07; (F) BP_03B04, (G) BP_03C05.

Target determination of chemical compound library #1 hits. In the A/T system poly(Phe) synthesis was inhibited by the seven chemical compound library #1 hits. Other than the ribosomes there are four accessory proteins that are required for poly(Phe) synthesis: 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 one of the accessory proteins was inhibited (see below).

First, the hit compounds were tested to determine if they inhibited the activity of PheRS. This assay tested the ability of PheRS to attach phenylalanine to its cognate $tRNA^{Phe}$. Purified *E. coli* $tRNA^{Phe}$ was used in these assays as described under "Methods and Materials". The results showed that only BP_03B04 inhibited the activity of PheRS (Fig 9).

Next, the ability of the hit compounds to inhibit the function of EF-Tu was tested. In the absence of EF-Ts and GTP, EF-Tu binds GDP and the exchange of the bound GDP for free GDP can be monitored. This binding has historically been used to characterize the activity of EF-Tu molecules from various species (64). In these assays [^{3}H]-labeled GDP was used to track the amount of GDP exchanged by EF-Tu and the ability of the test compounds to inhibit this exchange. We observed obvious reductions in the exchange of bound GDP by EF-Tu in the presence of BP_01C09 and BP_02H03 at concentration of 132 µM (Fig 10). In the presence of EF-Ts, the turnover of GDP binding by EF-Tu is stimulated 20-100 folds (65). Thus, when EF-Ts is added to the EF-Tu/GDP exchange assay in the presence of the test compounds, inhibition of the exchange stimulated by EF-Ts may be monitored. The concentration of EF-Tu was 1 µM and the time for completion of the assay was reduced from 30 min to 30 s. EF-Ts was added to a concentration equal to 5% of EF-Tu (0.05 µM). The stimulatory activity of EF-Ts was not affected by the presence of any of the test compounds (Fig 11).

We assayed the ribosome dependent GTPase activity of EF-G in order to determine the effect of the compounds on the function of EF-G. The amount of GTPase activity was determined by measurement of the amount of P_i liberated using a colorimetric GTPase assay kit (Novus Biologicals) as described under "Methods and Materials". None of the chemical compounds were detected to have the ability of inhibition of the GTPase activity of EF-G (Fig 12).

The remaining chemical compounds, BP_01F06, BP_02G06, BP_02G07 and BP_03C05 did not inhibit any of the accessory protein activities represented in the coupled aminoacylation/translation (A/T) screening assay. The inhibition of poly(Phe) synthesis in the

biochemical assay therefore leads us to conclude that the likely mechanism of action of these compounds is direct inhibition of the ribosome itself. The IC_{50} value and mechanism of action of chemical compound library #1 hits are listed in table 3.

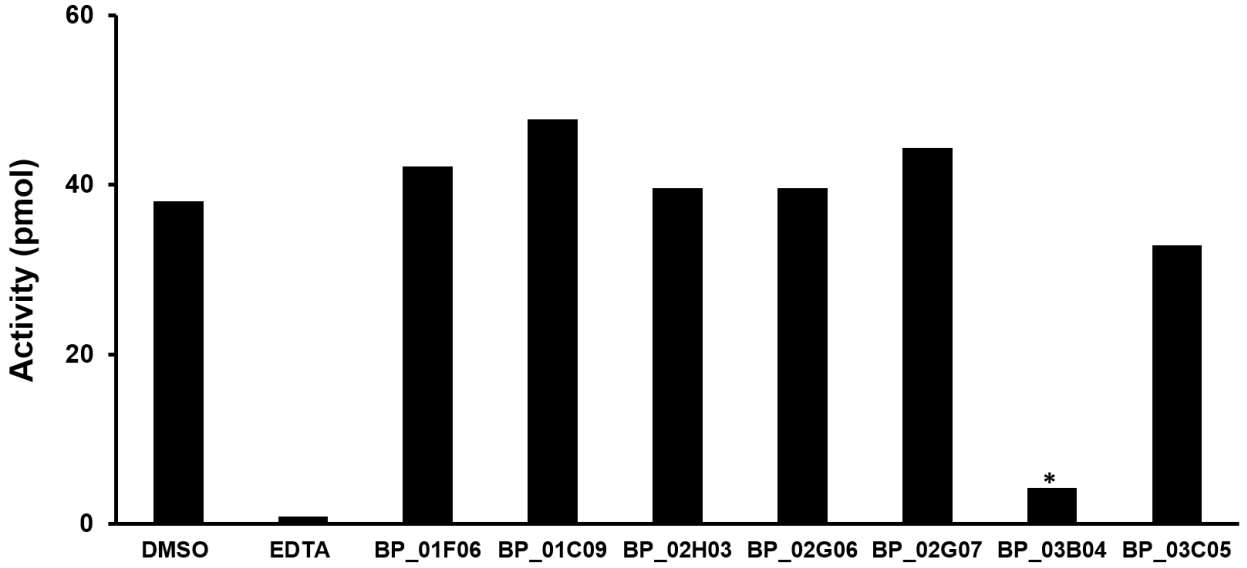


Fig 9. Results of PheRS assay of chemical compound library #1 hits. DMSO is the positive control, EDTA is the negative control. Compound that inhibited PheRS are labeled with an asterisk.

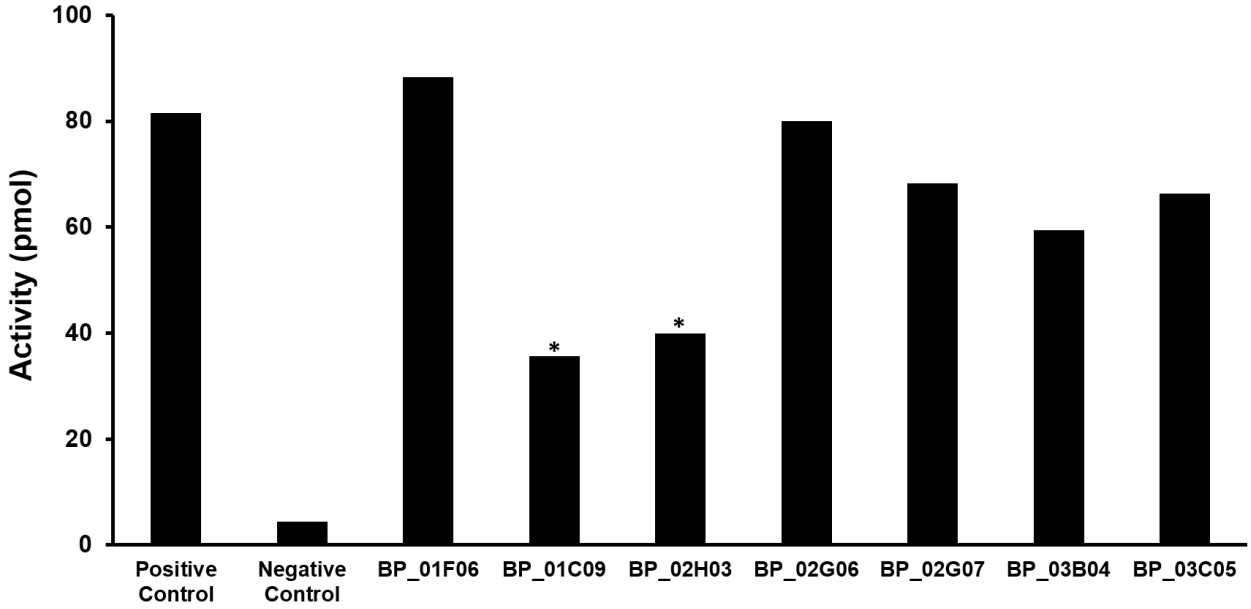


Fig 10. Results of EF-Tu assay of chemical compound library #1 hits. DMSO is the positive control, DMSO with no EF-Tu is the negative control. Compounds that inhibited EF-Tu were labeled with an asterisk.

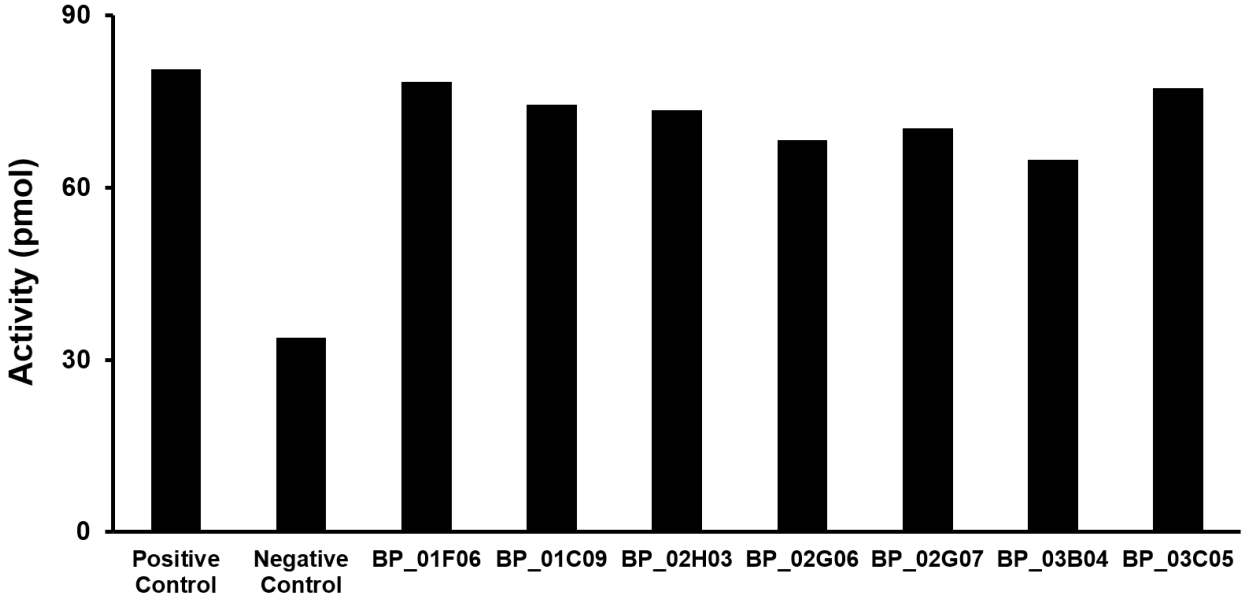


Fig 11. Results of EF-Ts assay of chemical compound library #1 hits. DMSO is the positive control, DMSO with no EF-Ts is the negative control.

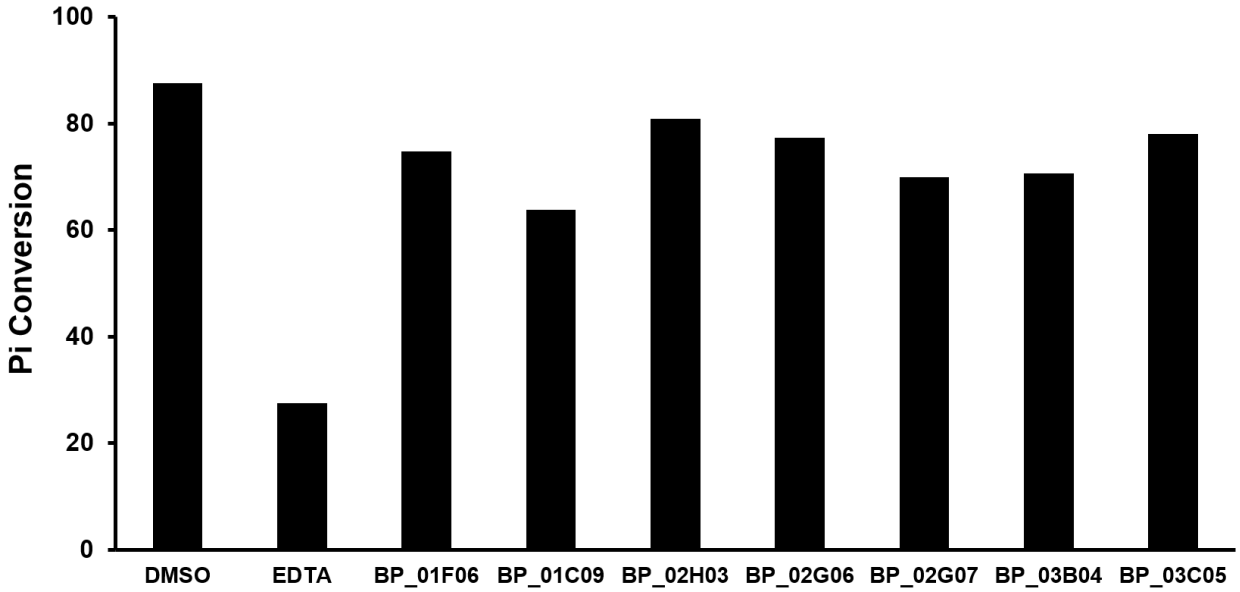


Fig 12. Results of EF-G assay of chemical compound library #1 hits. DMSO is the positive control, EDTA is the negative control.

Table 3. A/T system component inhibited and IC_{50} values for hit compounds of chemical compound library #1.

Chemical Compound	PheRS	EF-Tu	EF-Ts	EF-G	Ribosome	IC50 Value (μM)
BP_01F06					×	17.4
BP_01C09		×				31.5
BP_02H03		×				33.6
BP_02G06					×	41.3
BP_02G07					×	2.1
BP_03B04	×					4.9
BP_03C05					×	16.3

Next, the IC_{50} value for BP_03B04 in the PheRS assay was determined by serial dilution of the compound to a final concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.6, 0.8 and 0.4 μM in the PheRS assay. The effect of BP_03B04 on the activity of both *P. aeruginosa* and *S. pneumoniae* PheRS was tested and the results showed that BP_03B04 inhibited both *P. aeruginosa* and *S. pneumoniae* PheRS with IC_{50} value of 3.2 μM and 4.9 μM, respectively (Fig 13). This indicates that BP_03B04 may have broad spectrum activity.

Eukaryotic protein assays was used to determine whether BP_03B04 inhibited eukaryotic system. Wheat germ extract assays were used to determine whether it inhibited cytoplasmic protein synthesis. None inhibition was detected at concentrations of 132 μM (Fig 14A). BP_03B04 inhibited *P. aeruginosa* PheRS, therefore inhibition of human mitochondrial PheRS by this compound was determined and up to 75% of the activity of this system was observed to be inhibited at concentrations of 132 μM (Fig 14B).

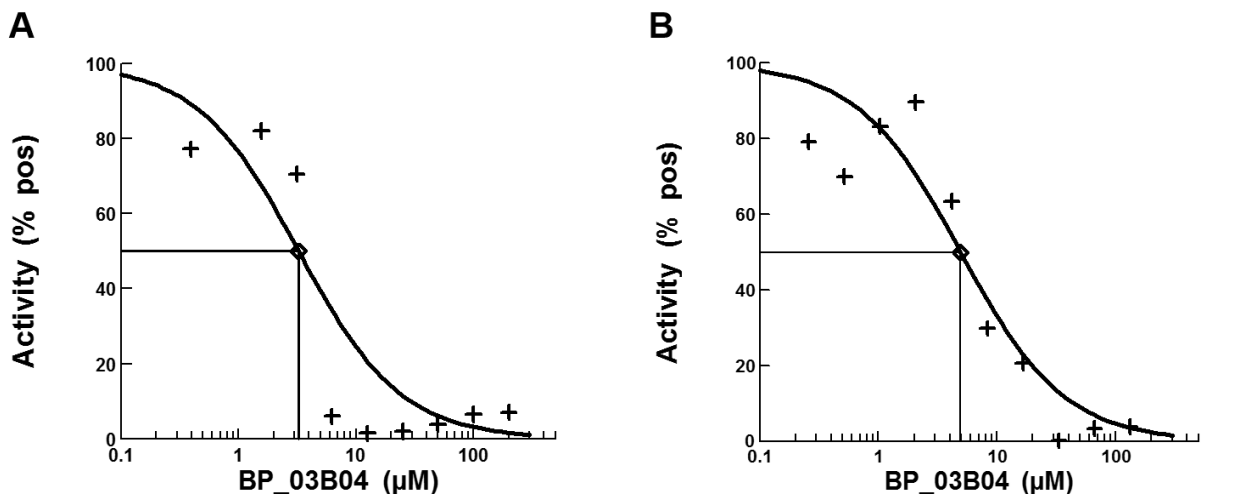


Fig 13. Determination of IC_{50} of BP_03B04 against *P. aeruginosa* and *S. pneumoniae* PheRS. A) IC_{50} of BP_03B04 against *P. aeruginosa* PheRS; B) IC_{50} of BP_03B04 against *S. pneumoniae* PheRS.

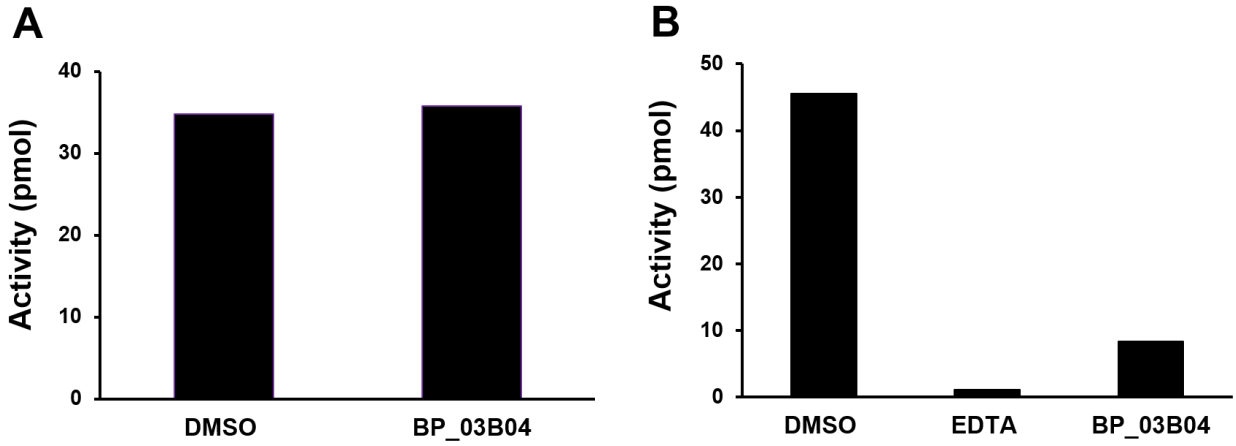


Fig 14. Eukaryotic protein assay of BP_03B04. A) Wheat germ extract assay. DMSO is the positive control; B) Human mitochondrial PheRS assay. DMSO is the positive control, EDTA is the negative control.

Microbiological testing of chemical compound library #1 hits. The chemical compound library #1 hits were tested in broth microdilution assays to determine minimum inhibitory concentrations (MIC). We tested the effect of the chemical compound library #1 hits on the growth of 10 pathogenic bacteria: *Moraxella catarrhalis* (ATCC 25238), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pneumonia* (ATCC 49619), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Haemophilus influenza* (49766), *Pseudomonas aeruginosa* (ATCC 47085) which were from the American Type Culture Collection (Manassas, VA). *P. aeruginosa Hypersensitive strain* (ATCC 35151), *P. aeruginosa PA200 Efflux* (efflux pump mutant), *E. coli TolC Efflux* (efflux pump mutant) were a kind gift from Urs Ochsner (Crestone Pharma-Boulder CO). Despite the similarity in biochemical activity of the compounds, the ability to inhibit bacterial growth varied widely.

Only BP_01F06 and BP_01C09 showed inhibition of *M. catarrhalis* at a concentration of 16 µg/ml and 64 µg/ml, separately. BP_02G07 showed inhibition of *S. aureus* at a concentration of 64 µg/ml. There were no inhibition at concentrations below 128 µg/ml by any of the other chemical compounds against any of the other bacteria tested. The MIC values of the chemical compound library #1 hits are listed in table 14.

Table 4. MIC values of the chemical compound library #1 hits.

Chemical Compound	MIC Value (µg/ml)									
	M. cat	*E. faecalis*	*S. pneumo*	*E. coli*	*S. aureus*	*H. flu*	*P. aeruginosa*	*P. aeruginosa Hypersensitive*	*P. aeruginosa PA200 Efflux*	*E. coli TolC Efflux*
BP_01F06	16	>128	128	>128	>128	>128	128	>128	>128	>128
BP_01C09	64	>128	128	>128	>128	NA	128	NA	>128	>128
BP_02H03	128	>128	128	>128	>128	NA	>128	>128	>128	>128
BP_02G06	128	>128	128	>128	>128	>128	128	128	>128	>128
BP_02G07	128	>128	128	NA	64	NA	NA	128	>128	>128
BP_03B04	>128	NA	128	NA	128	NA	128	NA	NA	NA
BP_03C05	128	>128	128	>128	>128	NA	128	NA	>128	>128

Screening of chemical compound library #2

Initial screening of chemical compound library #2. Using the optimized A/T system, the chemical compound library #2 containing 800 chemical compounds was next screened, as described in 'Materials and Methods'. The screening was the same format as described for chemical compound library #1. The position of the positive control (DMSO control), negative

control (EDTA control) and the chemical compounds were the same as the chemical compound library #1. The final concentration of the chemical compounds in this assay was also 132 µM. The results were plotted the same as with chemical compound library #1 on a 3-D scatter graph using JMP Pro 10.0.2 software. From the initial screening of chemical compound library #2, 55 primary hits, which had activity less than 50% of the DMSO control were identified (Fig 15).

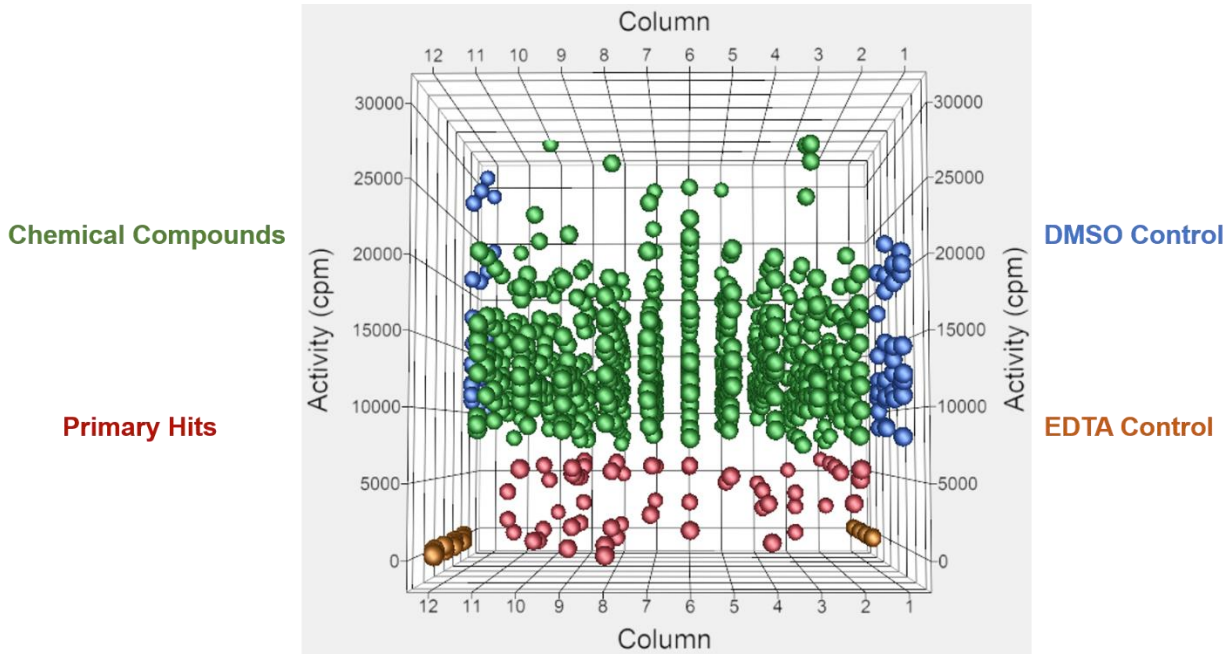


Fig 15. Initial screening results of chemical compound library #2. In the graph, the blue circles represent DMSO control, which is the positive control of the A/T assay; the orange circles represent EDTA control, which is the negative control of the A/T assay; the red circles represent our primary hits, which is below 50% activity of the positive control; the green circles represent all other chemical compounds in the chemical compound library #2.

Confirmation of chemical compound library #2 hits. The 55 primary hit compounds were re-assayed in duplicate in confirmation assays. From 55 primary hits, 36 compounds were confirmed to have inhibitory activity (Fig 16). The following work was based on these 36 hits.

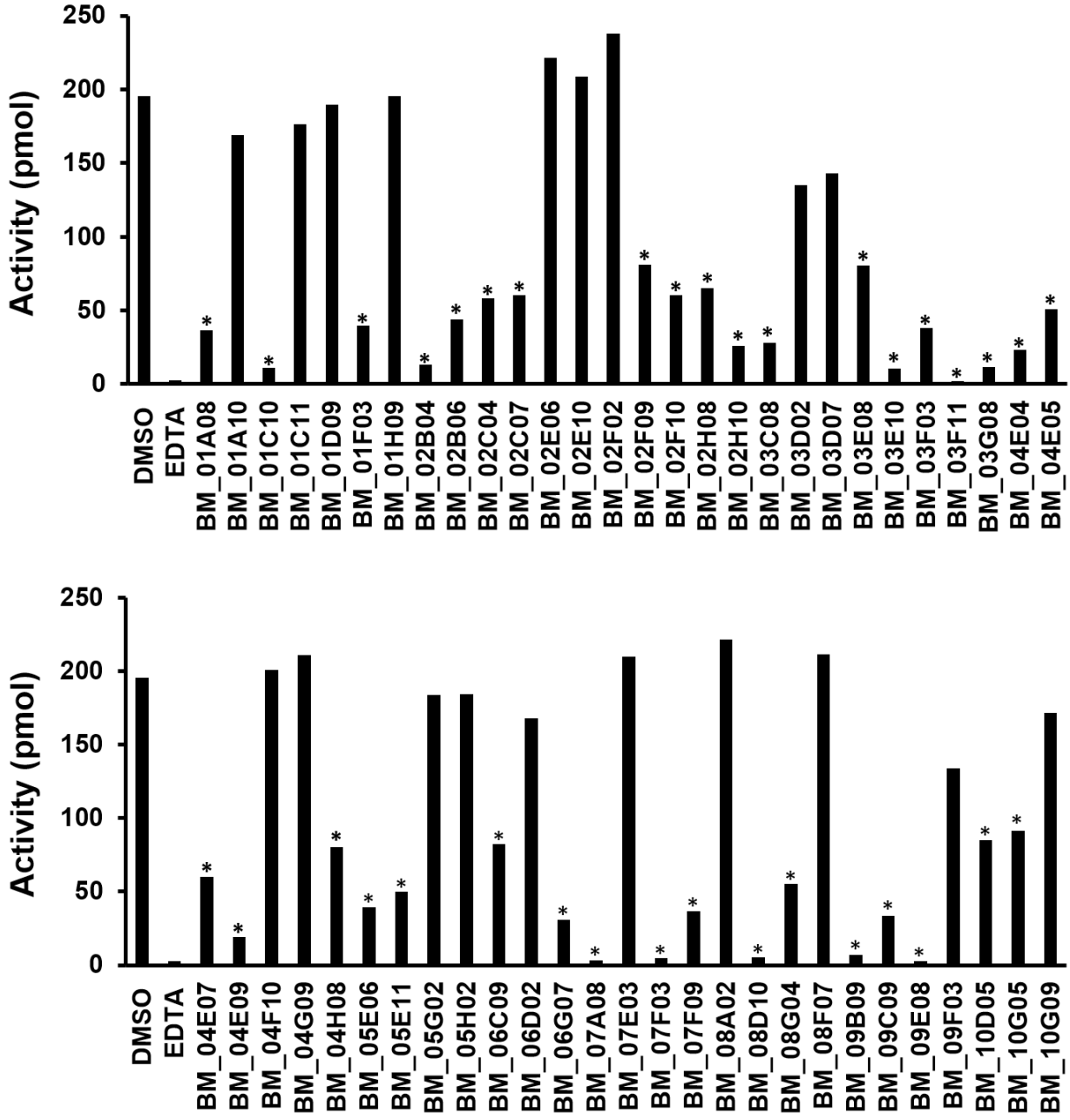
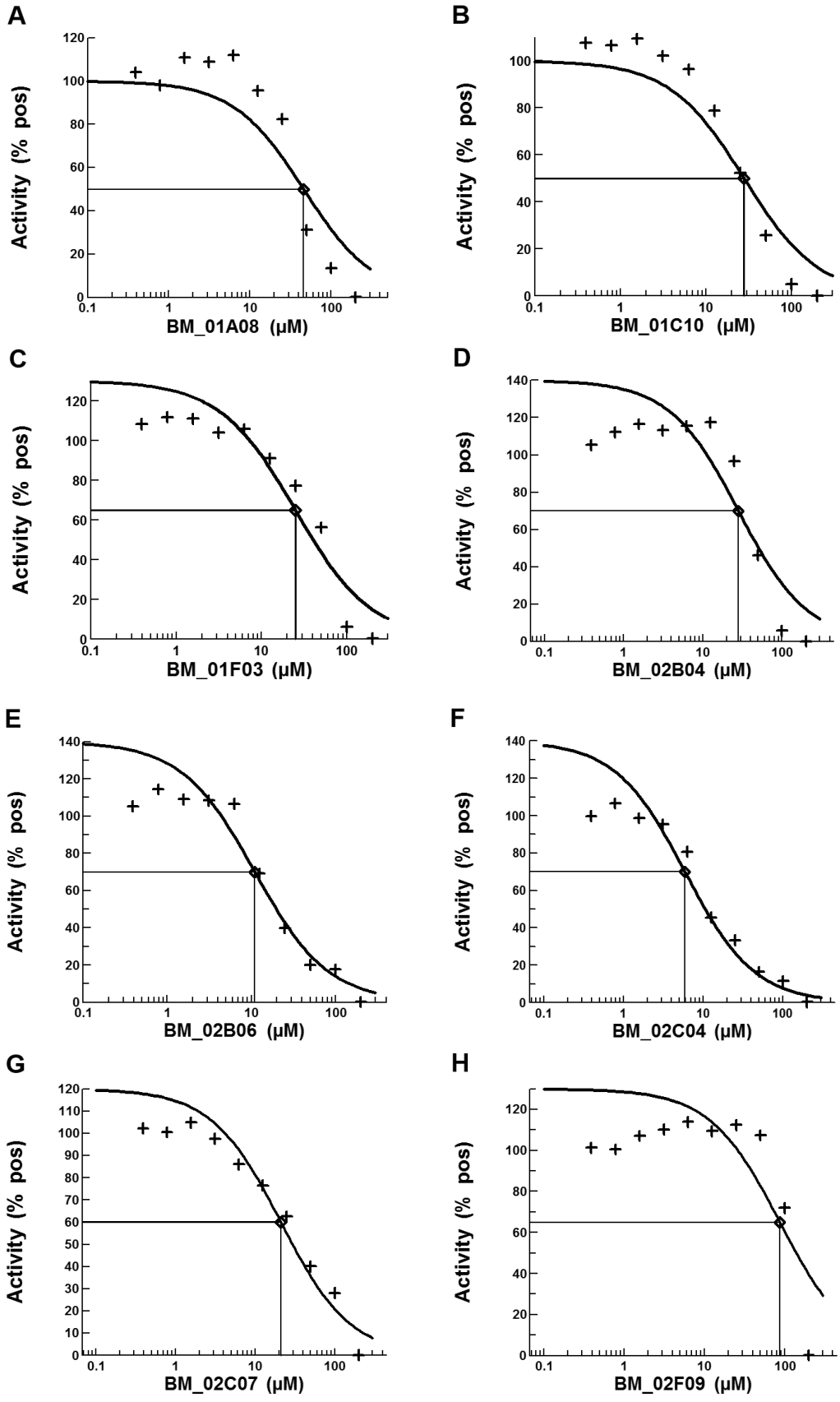
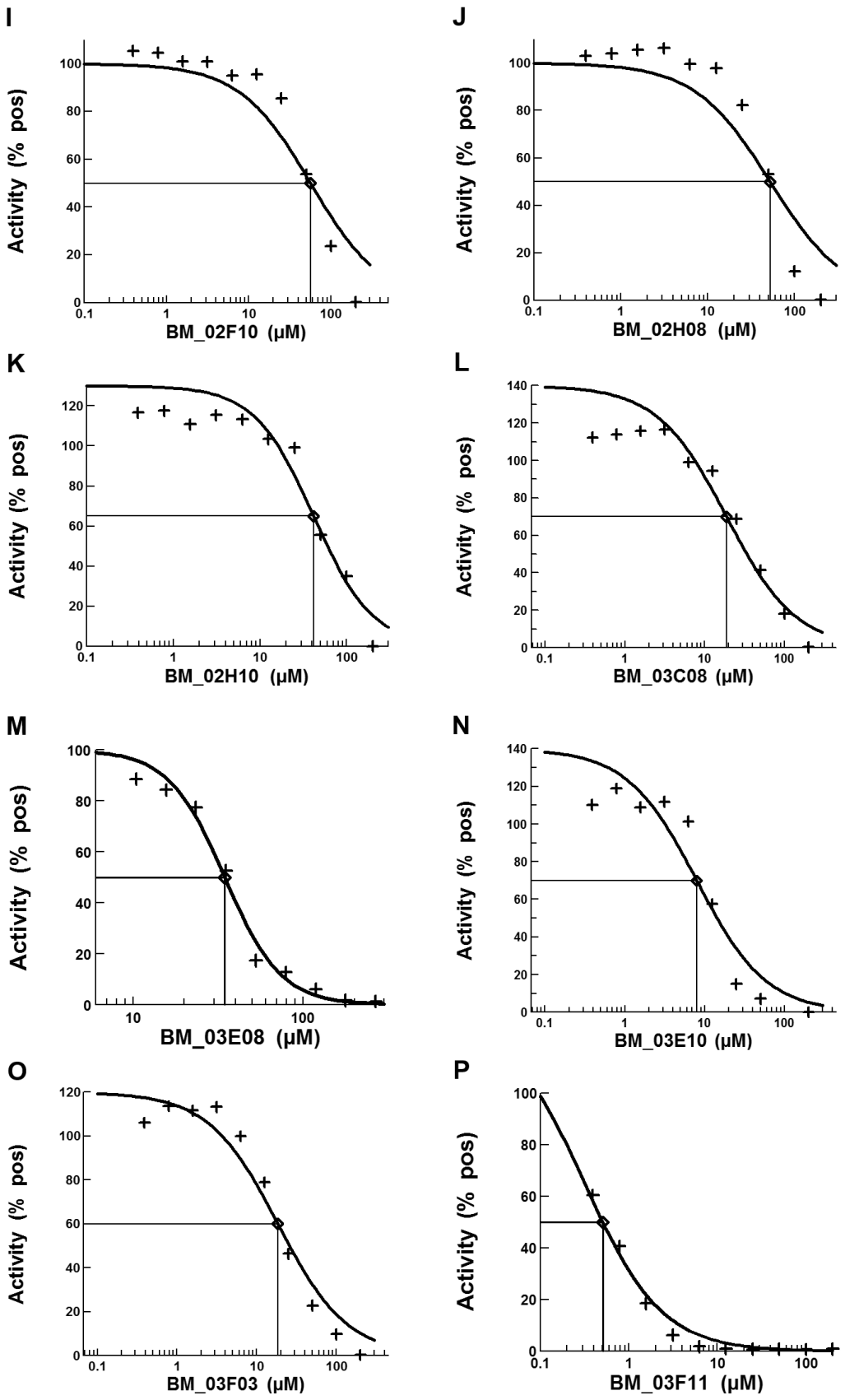


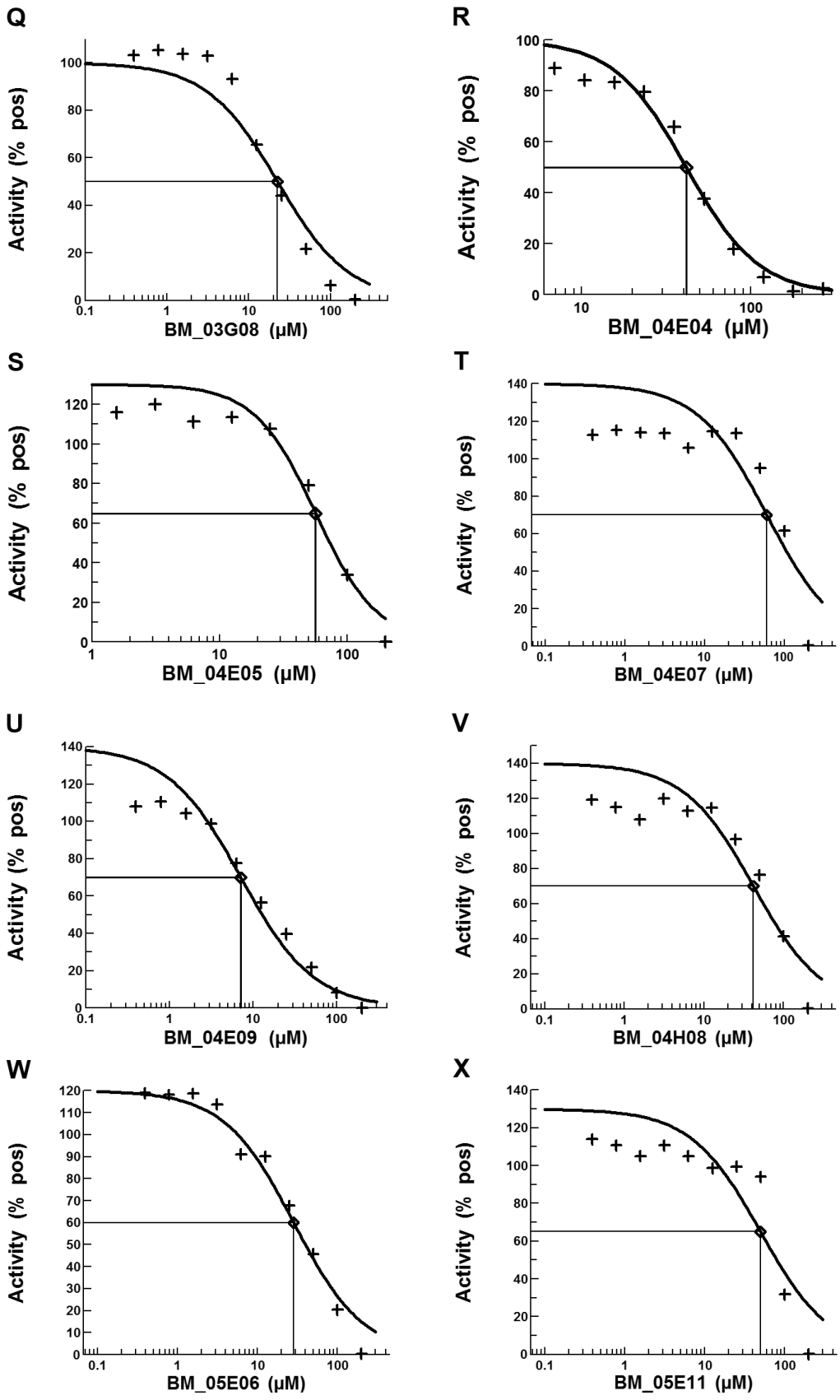
Fig 16. Confirmation of chemical compound library #2 hits. The confirmed hits are labeled with an asterisk.

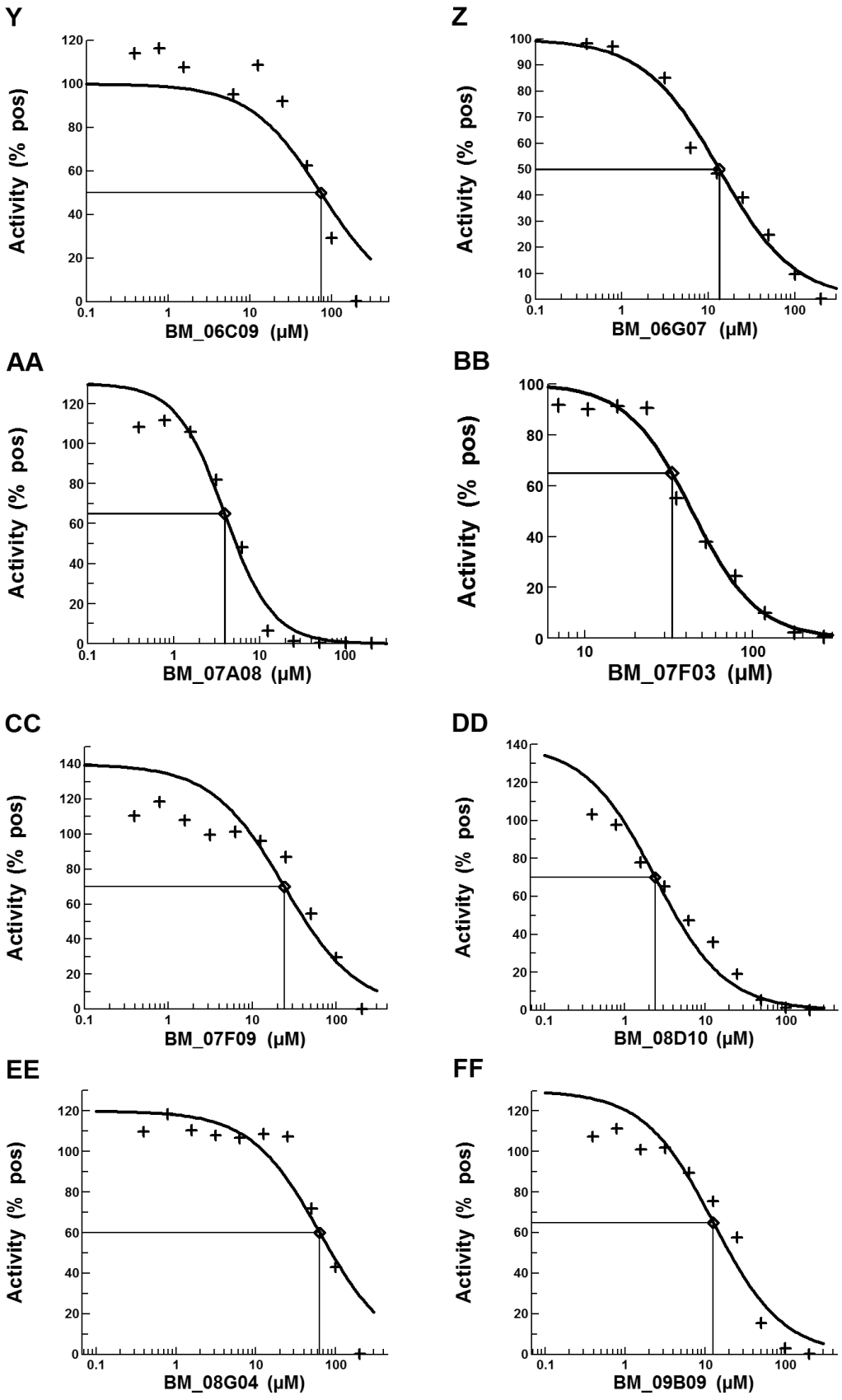
IC_{50} assay of chemical compound library #2 confirmed hits. Assays were carried out to determine the IC_{50} value of the confirmed hit compounds from library #2 using the same format as described for chemical compound library #1 (Fig 17).

The IC_{50} values of the chemical compound ranged from 0.31 to 87.2 µM, with BM_03F11 giving the lowest IC_{50} (0.31 µM), and BM_02F09 giving the highest IC_{50} (87.2 µM). The IC_{50} values for chemical compound library #2 are listed in table 4.


A
Activity (% pos)
BM_01A08 (μM)
B
Activity (% pos)
BM_01C10 (μM)
C
Activity (% pos)
BM_01F03 (μM)
D
Activity (% pos)
BM_02B04 (μM)
E
Activity (% pos)
BM_02B06 (μM)
F
Activity (% pos)
BM_02C04 (μM)
G
Activity (% pos)
BM_02C07 (μM)
H
Activity (% pos)
BM_02F09 (μM)


I
Activity (% pos)
BM_02F10 (μM)
J
Activity (% pos)
BM_02H08 (μM)
K
Activity (% pos)
BM_02H10 (μM)
L
Activity (% pos)
BM_03C08 (μM)
M
Activity (% pos)
BM_03E08 (μM)
N
Activity (% pos)
BM_03E10 (μM)
O
Activity (% pos)
BM_03F03 (μM)
P
Activity (% pos)
BM_03F11 (μM)


Q
Activity (% pos)
BM_03G08 (μM)
R
Activity (% pos)
BM_04E04 (μM)
S
Activity (% pos)
BM_04E05 (μM)
T
Activity (% pos)
BM_04E07 (μM)
U
Activity (% pos)
BM_04E09 (μM)
V
Activity (% pos)
BM_04H08 (μM)
W
Activity (% pos)
BM_05E06 (μM)
X
Activity (% pos)
BM_05E11 (μM)


Y
Activity (% pos)
BM_06C09 (μM)
Z
Activity (% pos)
BM_06G07 (μM)
AA
Activity (% pos)
BM_07A08 (μM)
BB
Activity (% pos)
BM_07F03 (μM)
CC
Activity (% pos)
BM_07F09 (μM)
DD
Activity (% pos)
BM_08D10 (μM)
EE
Activity (% pos)
BM_08G04 (μM)
FF
Activity (% pos)
BM_09B09 (μM)

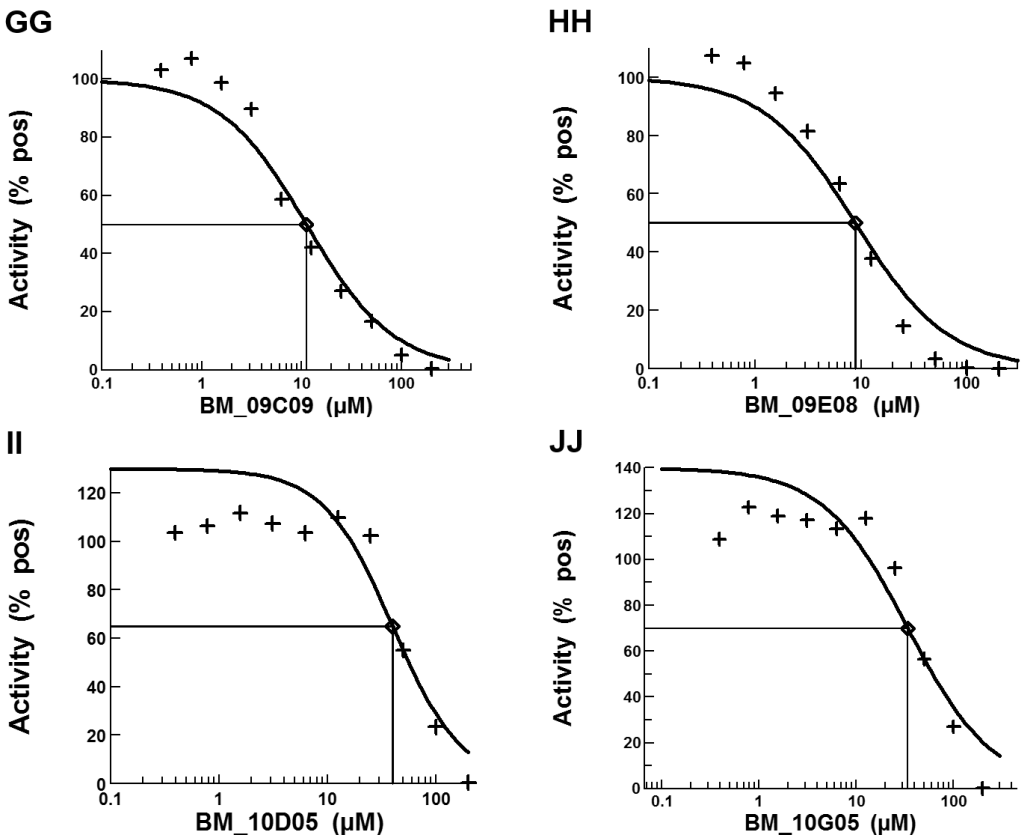


Fig 17. IC_{50} determination of the confirmed hits from chemical compound library #2. (A) BM_01A08; (B) BM_01C10; (C) BM_01F03; (D) BM_02B04; (E) BM_02B06; (F) BM_02C04; (G) BM_02C07; (H) BM_02F09; (I) BM_02F10; (J) BM_02H08; (K) BM_02H10; (L) BM_03C08; (M) BM_03E08; (N) BM_03E10; (O) BM_03F03; (P) BM_03F11; (Q) BM_03G08; (R) BM_04E04; (S) BM_04E05; (T) BM_04E07; (U) BM_04E09; (V) BM_04H08; (W) BM_05E06; (X) BM_05E11; (Y) BM_06C09; (Z) BM_06G07; (AA) BM_07A08; (BB) BM_07F03; (CC) BM_07F09; (DD) BM_08D10; (EE) BM_08G04; (FF) BM_09B09; (GG) BM_09C09; (HH) BM_09E08; (II) BM_10D05; (JJ) BM_10G05.

Target determination of chemical compound library #2 hits. In the A/T system poly(Phe) synthesis was inhibited by 36 chemical compounds from library #2. Specialty assays (PheRS assay, EF-Tu/EF-Ts GDP exchange assay and EF-G GTPase assay) were used to determine which of the accessory proteins was/were inhibited (see below).

The PheRS assay showed that compounds BM_01A08, BM_01C10, BM_01F03, BM_02B04, BM_02B06, BM_02C04, BM _02C07, BM_02F09, BM_02F10, BM_02H10, BM_03C08, BM_03E08, BM_03E10, BM_03G08, BM_04E04, BM_04E07, BM_04E09, BM_04H08, BM_05E11, BM_06C09, BM_07A08, BM_07F03, BM_07F09, BM_08D10, BM_08G04, BM_09C09, BM_09E08 and BM_10G05 inhibited the activity of PheRS (Fig 18).

Compound that are specific for only one accessory protein are of interest. Therefore, assays were carried out to determine if the compounds that inhibit PheRS also inhibit LeuRS. *P. aeruginosa* LeuRS has previously been purified in our laboratory (results not shown). This assay tested the ability of LeuRS to attach Leucine to its cognate $tRNA^{Leu}$. The results showed that compounds BM_01A08, BM_01C10, BM_01F03, BM_02B04, BM_02B06, BM_02C04, BM _02C07, BM_02F10, BM_02H10, BM_03C08, BM_03E10, BM_03G08, BM_04E07, BM_04E09, BM_04H08, BM_05E11, BM_06C09, BM_07A08, BM_07F09, BM_08D10, BM_08G04, BM_09C09 and BM_09E08 also inhibited LeuRS, and compounds BM_02F09, BM_03E08, BM_04E04, BM_07F03 and BM_10G05 had no effect on LeuRS (Fig 19).

Next, the EF-Tu/EF-Ts GDP exchange assay showed that compounds BM_01A08, BM_01F03, BM_02H10, BM_07A08, BM_09C09 and BM_09E08 inhibited EF-Tu (Fig 20), and none of the chemical compounds inhibited EF-Ts (Fig 21). Finally, the EF-G GTPase assay showed that compounds BM_01A08, BM_02B04, BM_02B06, BM_03E10, BM_03F11, BM_03G08, BM_07A08, BM_07F09, BM_08D10 and BM_09E08 inhibited EF-G (Fig 22).

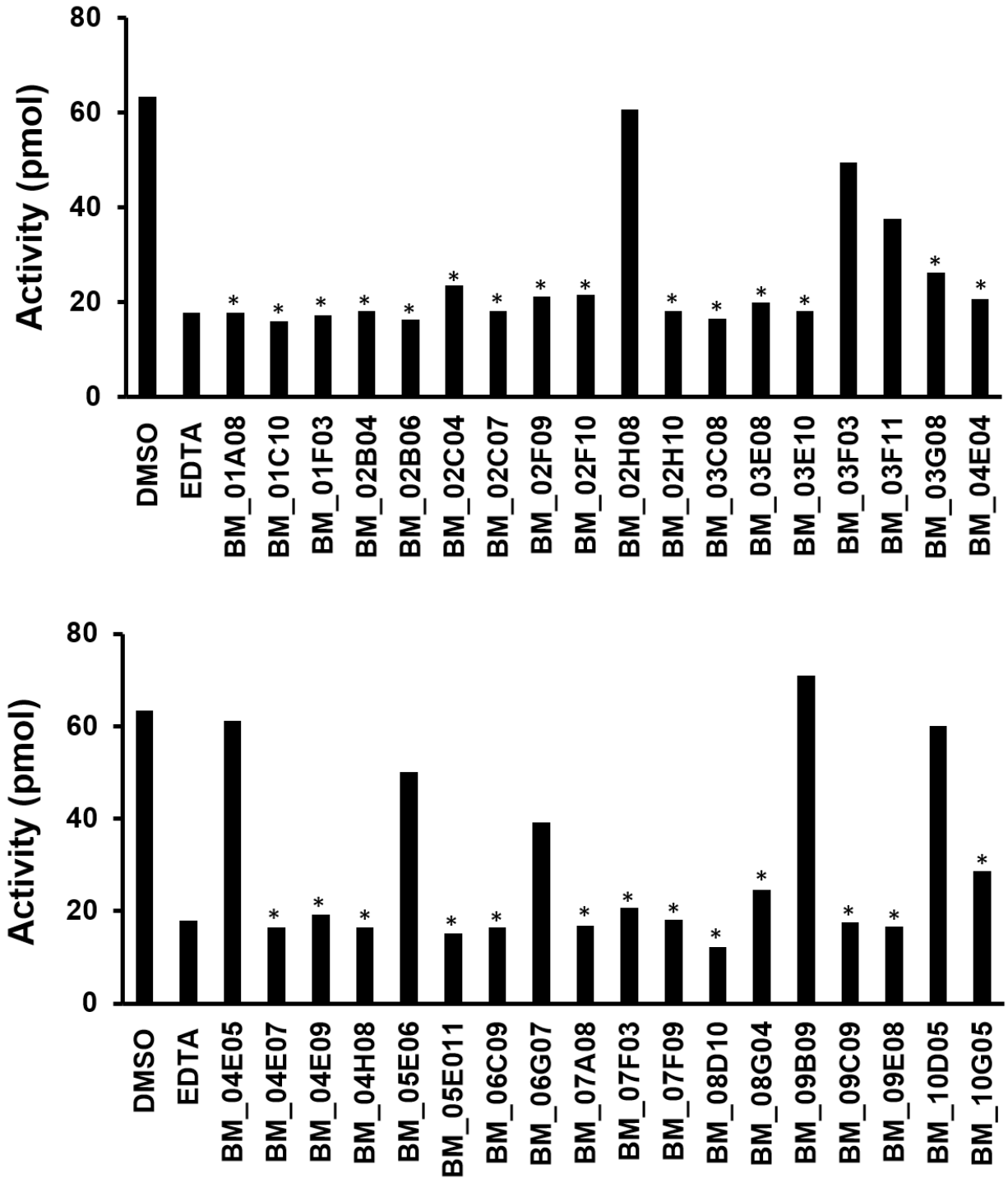


Fig 18. PheRS assay of chemical compound library #2 hits. DMSO is the positive control, EDTA is the negative control. Compounds that inhibited PheRS are labeled with an asterisk.

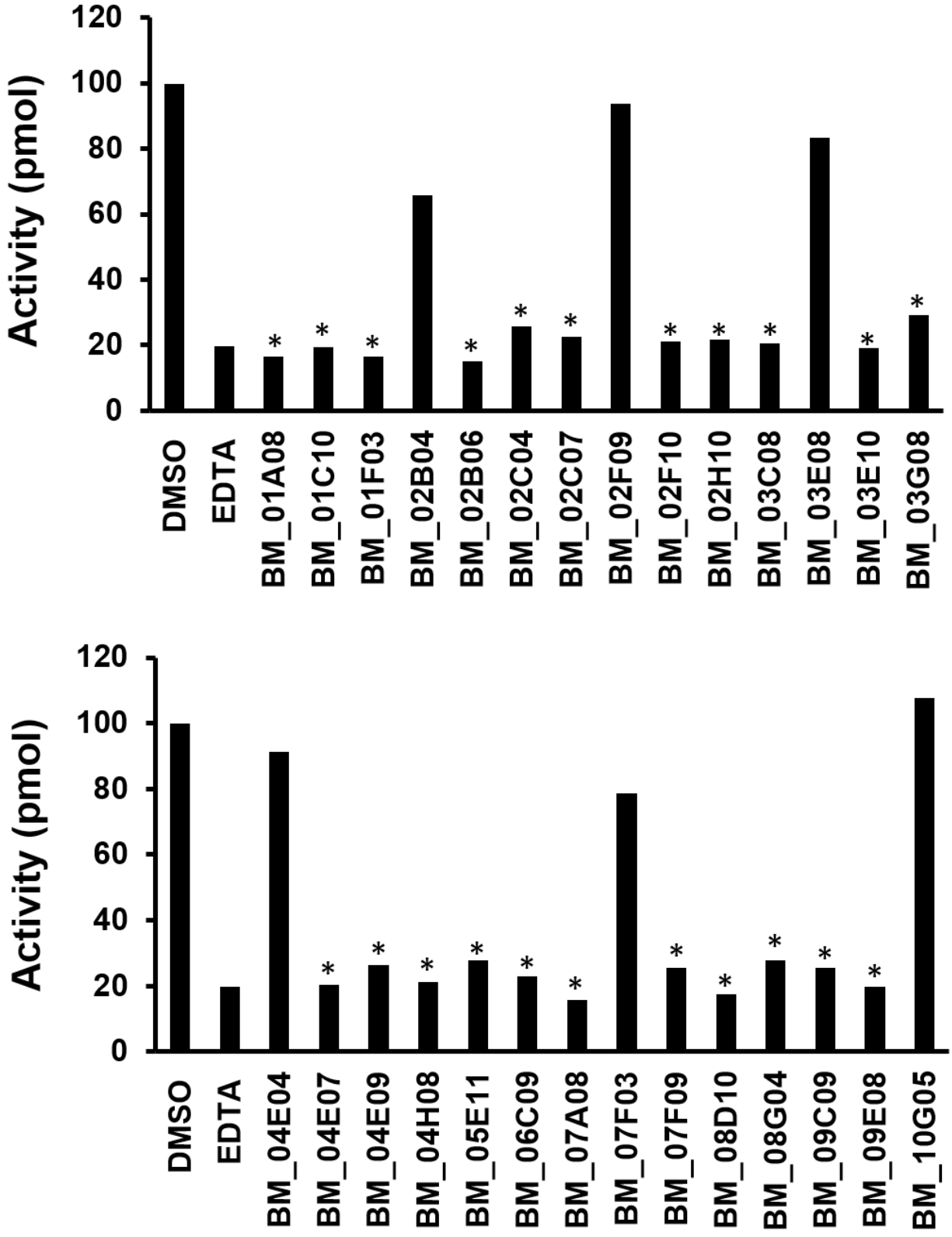


Fig 19. LeuRS assay of chemical compound library #2 hits. DMSO is the positive control, EDTA is the negative control. Compounds that inhibited LeuRS are labeled with an asterisk.

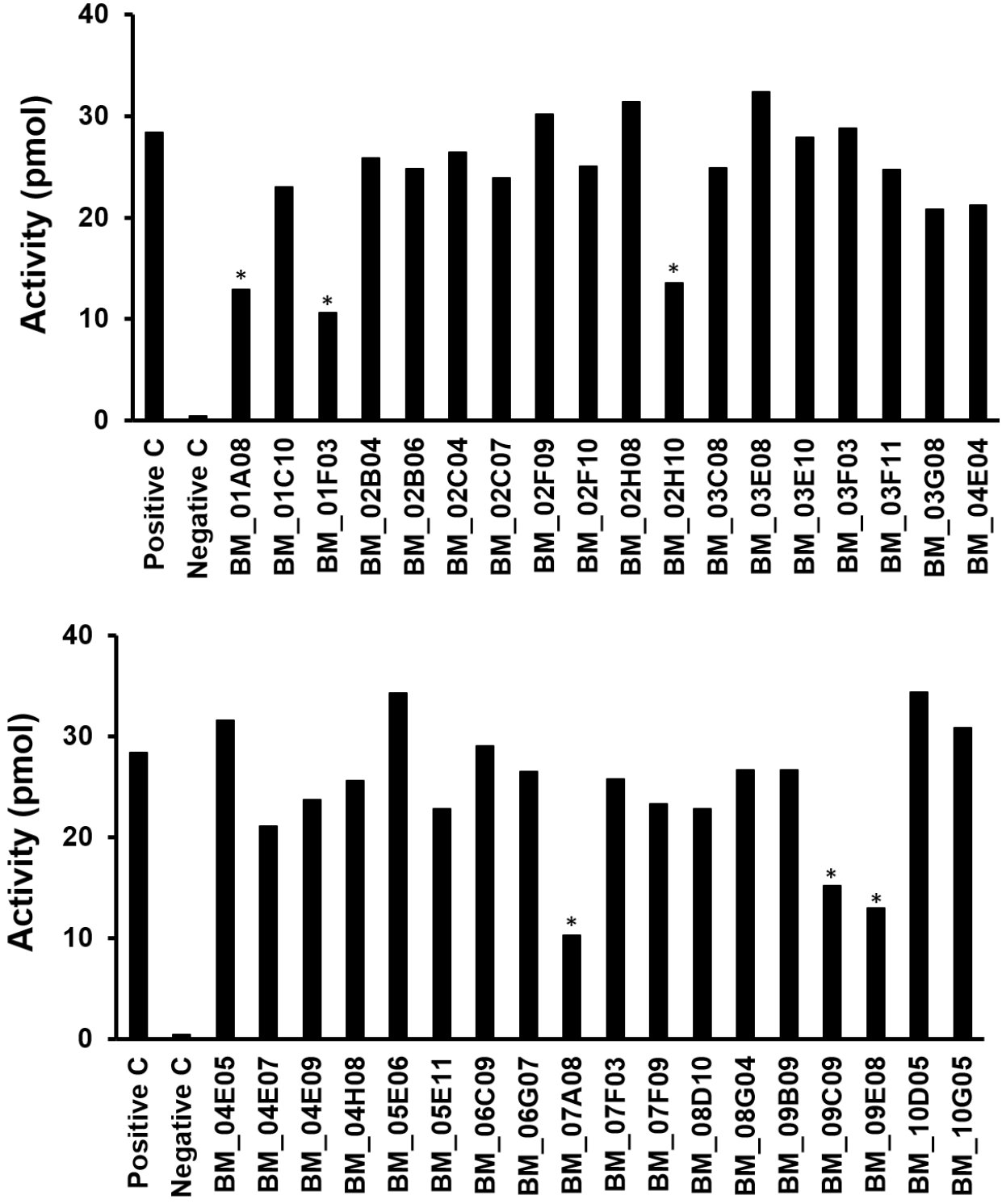


Fig 20. EF-Tu assay of chemical compound library #2 hits. DMSO is the positive control, DMSO with no EF-Tu is the negative control. Compounds that inhibited EF-Tu are labeled with an asterisk.

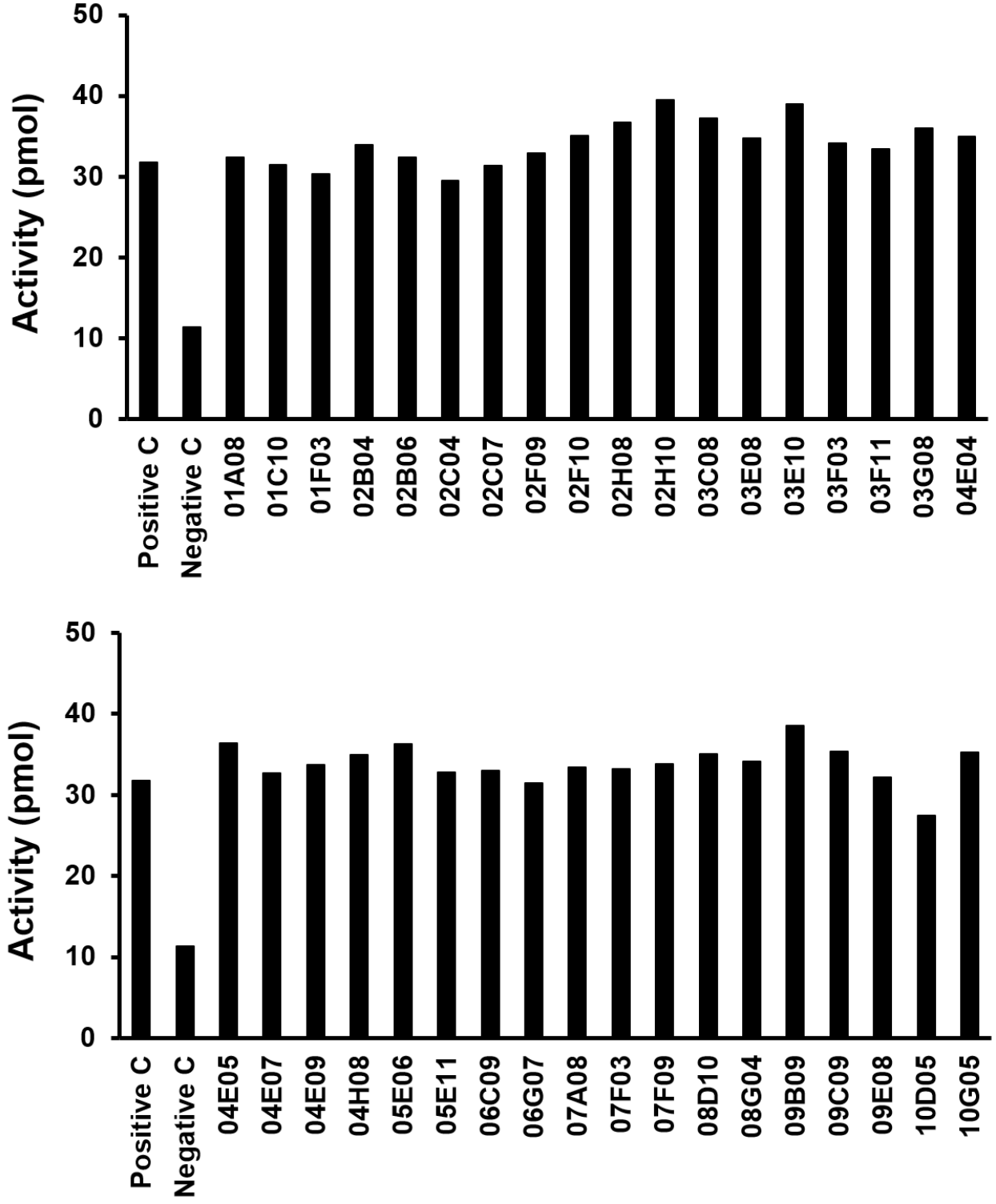


Fig 21. EF-Ts assay of chemical compound library #2 hits. DMSO is the positive control, DMSO with no EF-Ts is the negative control.

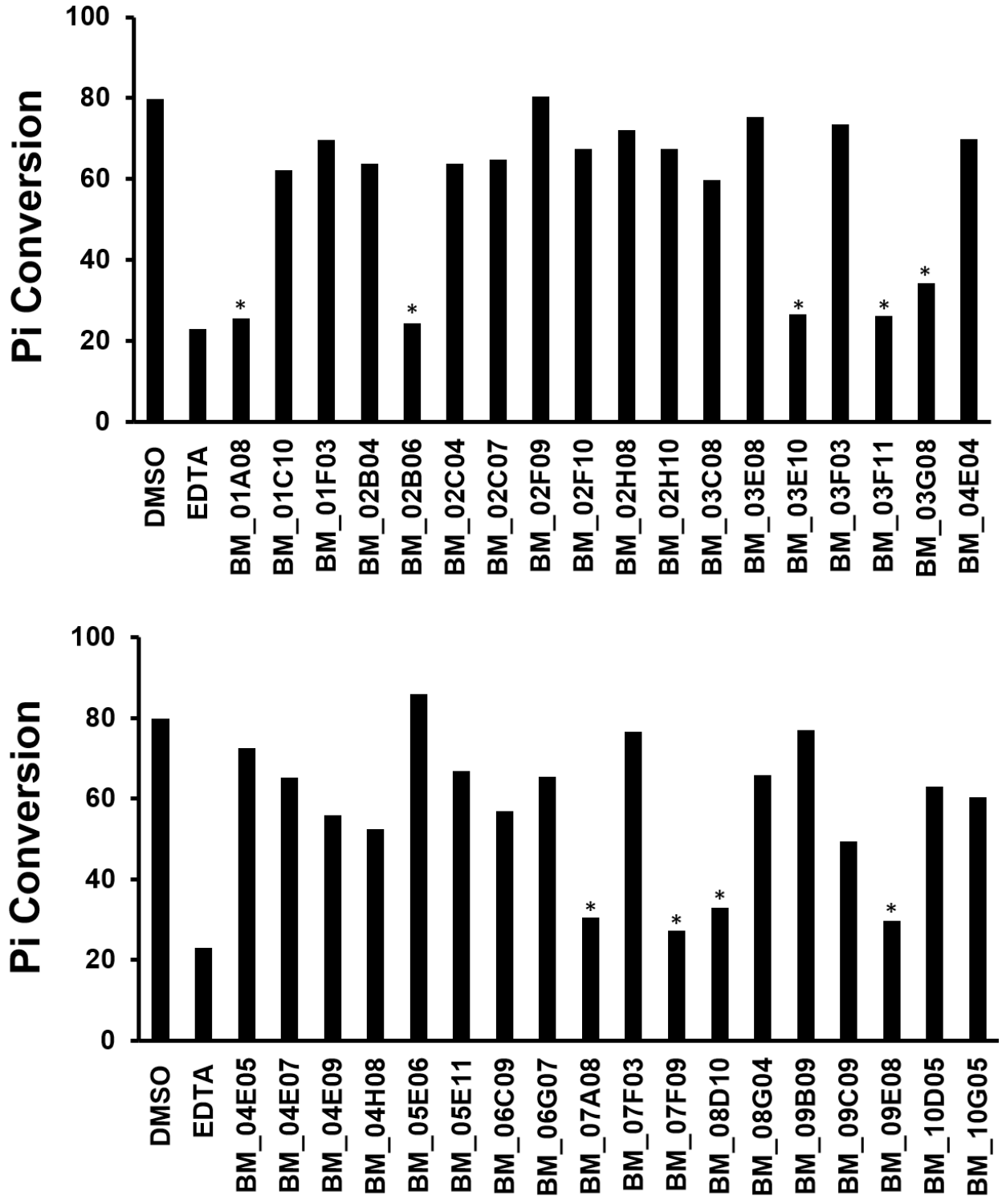


Fig 22. EF-G assay of chemical compound library #2 hits. DMSO is the positive control, EDTA is the negative control. Compounds that inhibited EF-G are labeled with an asterisk.

The remaining chemical compounds, BM_02H08, BM_03F03, BM_04E05, BM_05E06, BM_06G07, BM_09B09 and BM_10D05 did not inhibit any of the accessory protein activities represented in the coupled aminoacylation/translation (A/T) screening assay. The inhibition of poly(Phe) synthesis in the biochemical assay therefore leads us to conclude that the likely mechanism of action of these compounds is direct inhibition of the ribosome itself.

The mechanism of action of chemical compound library #2 hits are listed in table 5. From the table, only 13 chemical compounds out of the original 36 compounds (labeled with an asterisk on the top right): BM_02F09, BM_02H08, BM_03E08, BM_03F03, BM_03F11, BM_04E04, BM_04E05, BM_05E06, BM_06G07, BM_07F03, BM_09B09, BM_10D05, BM_10G05 were observed to have unique targets. These targets have been identified as EF-G, PheRS or ribosomes. The following work focused on these 13 chemical compounds.

Table 5. A/T system component inhibited and IC_{50} values for hit compounds of chemical compound library #2.

Chemical Compounds	EF-G	EF-Tu	EF-Ts	PheRS	LeuRS	Ribosome	IC50 Value (μM)
BM_01A08	×	×		×	×		45.7
BM_01C10				×	×		27.7
BM_01F03		×		×	×		24.9
BM_02B04	×			×			28.1
BM_02B06	×			×	×		10.9
BM_02C04				×	×		5.8
BM_02C07				×	×		21
BM_02F09*				×			87.2
BM_02F10				×	×		56.5
BM_02H08*						×	52.2
BM_02H10		×		×	×		41.5
BM_03C08				×	×		18.7
BM_03E08*				×			34.5
BM_03E10	×			×	×		7.9
BM_03F03*						×	18.3
BM_03F11*	×						0.31
BM_03G08	×			×	×		22.4
BM_04E04*				×			42.2
BM_04E05*						×	56.6
BM_04E07				×	×		60.3
BM_04E09				×	×		7.0
BM_04H08				×	×		41.7
BM_05E06*						×	28.3
BM_05E11				×	×		49.7
BM_06C09				×	×		74.3
BM_06G07*						×	13.3
BM_07A08	×	×		×	×		3.9
BM_07F03*				×			33.0
BM_07F09	×			×	×		24
BM_08D10	×			×	×		2.3
BM_08G04				×	×		63.2
BM_09B09*						×	12.6
BM_09C09		×		×	×		11.1
BM_09E08	×	×		×	×		8.8
BM_10D05*						×	39.9
BM_10G05*				×			34.0

BM_03F11 was the only hit compound observed to inhibit EF-G. The IC_{50} value for BM_03F11 in the EF-G assay was determined using serial dilutions of the compound to a final concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.6, 0.8 and 0.4 µM in the EF-G assay. The effect of BM_03F11 on the activity of both *P. aeruginosa* and *B. subtilis* EF-G was tested and the results showed that BM_03F11 inhibited both *P. aeruginosa* and *B. subtilis* EF-G with IC_{50} value of 12.0 µM and 3.6 µM, respectively (Fig 23). This indicates that BM_03F11 may have broad spectrum activity.

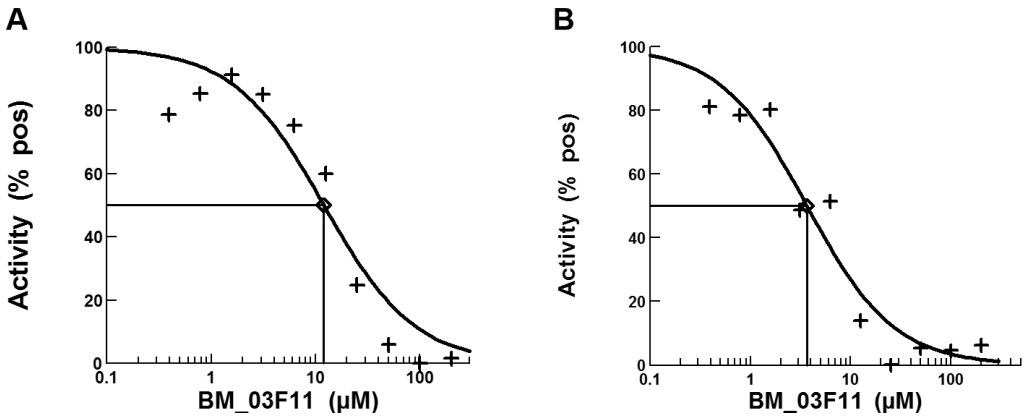


Fig 23. Determination of IC_{50} of BM_03F11 against *P. aeruginosa* and *B. subtilis* EF-G.

The IC_{50} values of compounds that inhibited PheRS (BM_02F09, BM_03E08, BM_04E04, BM_07F03, BM_10G05) were determined using the same serial dilution (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.6, 0.8 and 0.4 µM) of compounds in the PheRS assay (Fig 24). The IC_{50} value of these compounds ranged from 17.4 to 54.6 µM (Table 6), with BM_04E04 having the lowest IC_{50} of 17.4 µM and BM_10G05 having the highest IC_{50} of 54.6 µM.

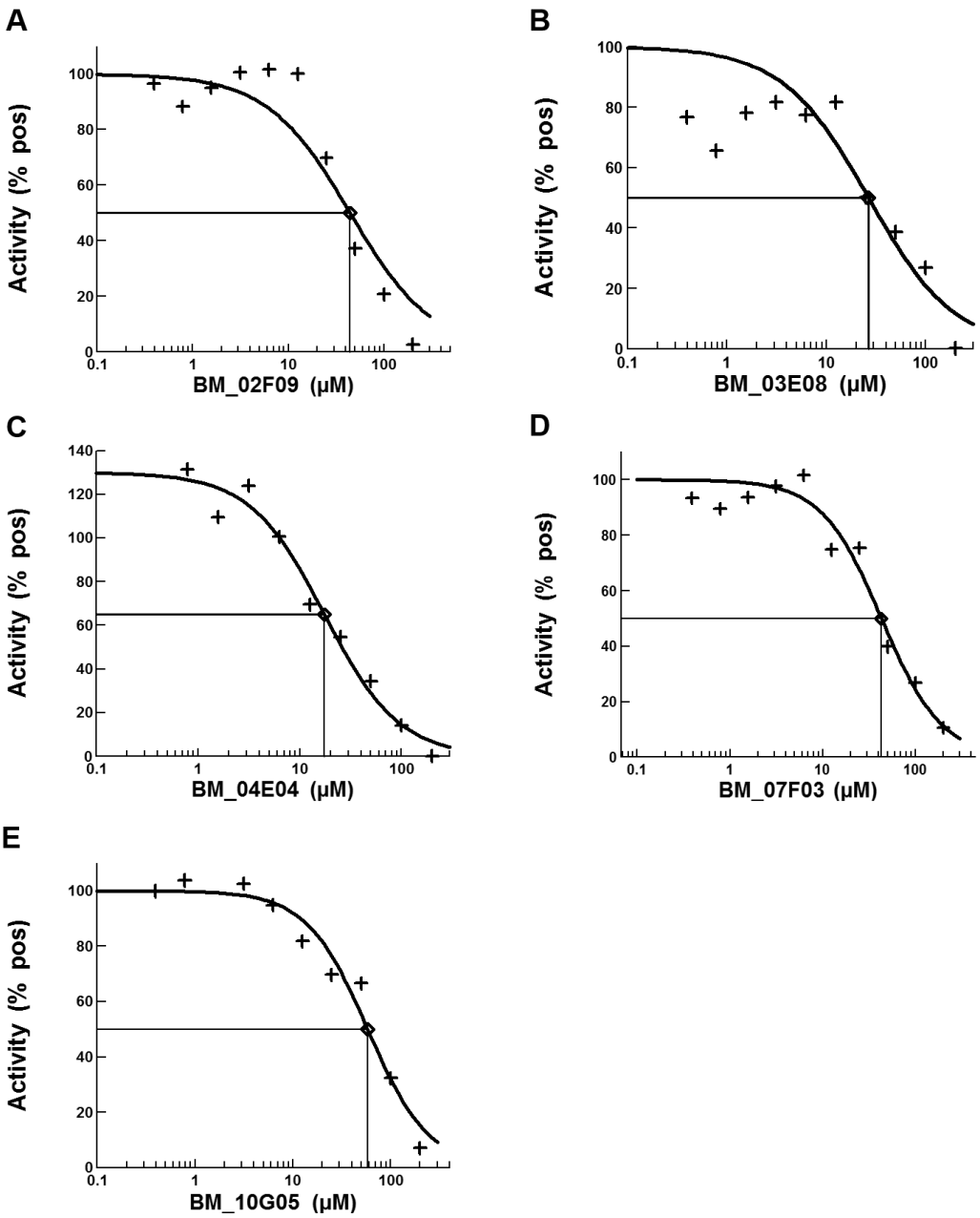


Fig 24. Determination of IC_{50} of chemical compounds from library #2 that inhibit PheRS in *P. aeruginosa* PheRS assays.

Table 6. IC_{50} value of chemical compounds that inhibited *P. aeruginosa* PheRS in the PheRS assay.

Chemical Compound	IC50 Value (µM)
BM_02F09	44.2
BM_03E08	26.5
BM_04E04	17.4
BM_07F03	43.1
BM_10G05	54.6

Microbiological testing of hit chemical compounds from library #2. The 13 inhibitors with activity against specific components of the A/T system were tested in broth microdilution assays to determine minimum inhibitory concentrations (MIC) as described previously for hit chemical compounds from library #1. The effect of these 13 hit compounds on the growth of 10 pathogenic bacteria were determined and the results are listed in Table 7.

Table 7. MIC value of chemical compound library #2 inhibitors.

Chemical Compound	MIC Value (µg/ml)									
	M. cat	*E. faecalis*	*S. pneumo*	*E. coli*	*S. aureus*	*H. flu*	*P. aeruginosa*	*P. aeruginosa Hypersensitive*	*P. aeruginosa PA200 Efflux*	*E. coli TolC Efflux*
BM_02F09	32	>128	64	>128	128	32	128	4	>128	64
BM_02H08	>128	>128	128	>128	>128	32	>128	4	>128	>128
BM_03E08	16	16	64	>128	128	16	128	16	>128	>128
BM_03F03	>128	>128	128	>128	>128	>128	>128	128	>128	>128
BM_03F11	128	>128	128	>128	64	4	128	8	>128	>128
BM_04E04	1	8	64	>128	128	32	128	8	>128	16
BM_04E05	128	>128	128	>128	128	>128	>128	128	>128	>128
BM_05E06	>128	>128	>128	>128	>128	128	128	>128	>128	>128
BM_06G07	64	>128	>128	>128	128	32	>128	>128	>128	>128
BM_07F03	16	32	64	>128	128	16	>128	16	>128	2
BM_09B09	>128	64	128	>128	>128	32	16	32	>128	>128
BM_10D05	128	>128	128	>128	>128	>128	128	>128	>128	>128
BM_10G05	>128	>128	>128	>128	>128	>128	128	>128	>128	>128

Surprisingly, none of the chemical compounds were observed to inhibit the growth of *P. aeruginosa PA200 Efflux* (efflux pump mutant) or wild type *E. coli* lower than the concentration of 128 µg/ml. However, BM_02F09, BM_04E04 and BM_07F03 inhibited *E. coli TolC Efflux*

(efflux pump mutant) at a concentration of 64 µg/ml, 16 µg/ml and 2 µg/ml, respectively. Most of the chemical compounds had very good effect on the *P. aeruginosa* hypersensitive strain. For example, BM_02F09, BM_02H08, BM_03E08, BM_03F11, BM_04E04, BM_07F03 and BM_09B09 had MIC values of 4 µg/ml, 4 µg/ml, 16 µg/ml, 8 µg/ml, 8 µg/ml, 16 µg/ml and 32 µg/ml, respectively. Only compound BM_09B09 worked well against wild type *P. aeruginosa*, and resulting in an MIC value of 16 µg/ml. None of the rest of the chemical compounds inhibit *P. aeruginosa* growth at a concentration lower than 128 µg/ml.

Many of the chemical compounds inhibited growth of *M. catarrhalis, E. faecalis, S. pneumonia*, *S. aureus*, and *H. influenza*. For example, BM_02F09, BM_03E08, BM-04E04 and BM_06G07 had good MIC values against *M. catarrhalis* of 32 µg/ml, 16 µg/ml, 1 µg/ml, 64 µg/ml, and 16 µg/ml respectively. For *E. faecalis*, BM_03E08, BM_04E04, BM_07F03 and BM_09B09 had the MIC values of 16 µg/ml, 8 µg/ml, 32 µg/ml and 64 µg/ml respectively. Compounds BM_02F09, BM_02H08, BM_03E08, BM_03F11, BM_04E04, BM_06G07, BM_07F03 and BM_09B09 inhibited *H. influenza* at the concentrations of 32 µg/ml, 32 µg/ml, 16 µg/ml, 4 µg/ml, 32 µg/ml, 32 µg/ml, 16 µg/ml and 32 µg/ml respectively. However, cultures of *S. pneumonia* and *S. aureus* were not inhibited as well as that of *M. catarrhalis*, *E. faecalis* and *H. influenza*. Only BM_02F09, BM_03E08, BM_04E04 and BM_07F03 inhibited growth of *S. pneumonia*. These compounds all inhibited *S. pneumoniae* with MIC values of 64 µg/ml. Only compound BM_03F11 was observe to inhibit *S. aureus*, and the MIC was at a concentration of 64 µg/ml. There were none of the remaining compounds that inhibited this bacteria at concentrations lower than 128 µg/ml.

Overall, compounds BM_03E08, BM_04E04 and BM_07F03 showed the highest activity against most of the pathogenic bacteria tested, these results indicate that they may have broad spectrum activity. Compounds BM_03F03, BM_04E05, BM_05E06, BM_10D05 and BM_10G05 did not inhibit any of the bacteria at the concentrations lower than 128 µg/ml.

Time kill study. Next, time kill studies were performed using the three compounds (BM_03E08, BM_04E04 and BM_07F03) observed to have the greatest levels of inhibition in the MIC studies to determine whether they are bacteriostatic or bactericidal. One Gram-negative bacteria *M. catarrhalis* (Fig 25) and one Gram-positive bacteria *E. faecalis* (Fig 26) were chosen to be tested against these compounds.

BM_03E08 was shown to completely inhibited *M. catarrhalis* growth at 2, 4 and 6 hours, but the bacteria was observed to product colonies at 24 hours. This was likely due to the lack of complete inhibition of bacterial growth in the early phase (2, 4 and 6 hours). There were apparently some bacteria cell still viable, therefore at 24 hours bacteria growth was observed. These results suggest that BM_03E08 is a bacteriostatic agent against *M. catarrhalis*.

BM_04E04 also did not kill the *M. catarrhalis* cells in the assays. The bacterial growth was similar to that of the control (without any inhibitor) at 2 and 4 hours, but at 6 and 24 hours growth was limited compare to the control. At 6 hours, fewer colonies were observed than at 4 hours, and at 24 hours colony forming units (CFU) was further decreased. Only 4 µg/ml BM_04E04 was present in these assays. These results suggest that BM_04E04 may be a bacteriostatic agent at lower concentrations and a bactericidal agent at higher concentrations against *M. catarrhalis*.

BM_07F03 killed all the *M. catarrhalis* cells at all the time assayed, from 2 to 24 hours. This indicates that it is a bactericidal agent against *M. catarrhalis*.

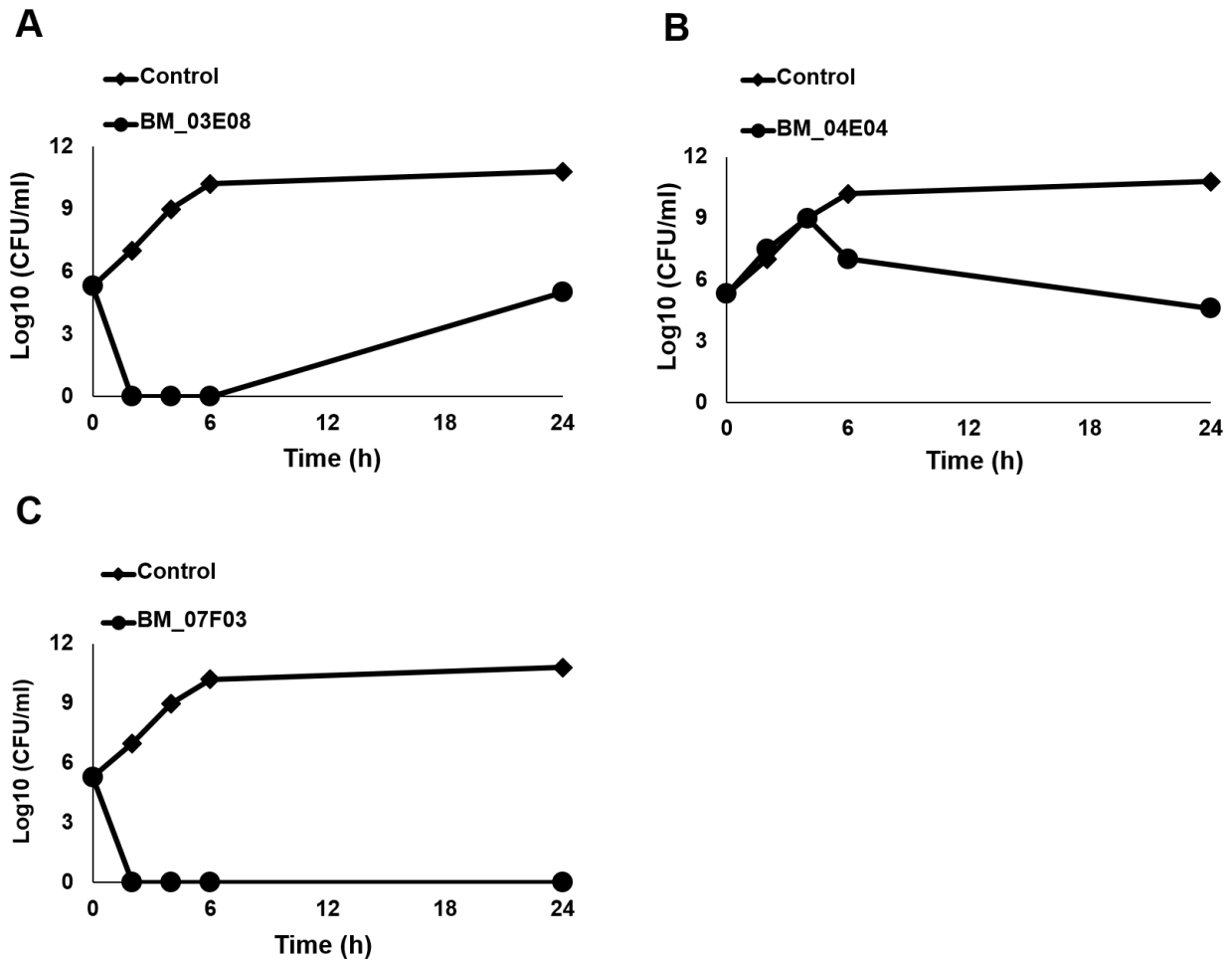


Fig 25. Time-kill kinetics of compound hits from library #2 against *M. catarrhalis*. Compound hits were added to bacterial cultures at four times the MIC. Samples were analyzed by plating and determination of colony forming units (CFU) at 0, 2, 4, 6, and 24 h. (A) BM_03E08; (B) BM_04E04; (C) BM_07F03. Diamonds (♦) represent control cultures grown in the absence of inhibitor, and filled circles (●) represent cultures containing the tested compounds at 4×MIC.

BM_03E08 and BM_04E04 did not kill *E. faecalis* cells in the assays up to 24 hours. There were no obvious increase or decrease in viable colonies with the colony number remaining constant. These results indicate that BM_03E08 and BM_04E04 are bacteriostatic agents against *E. faecalis*. Alternatively, BM_07F03 appeared to kill *E. faecalis* cells throughout all the times, from 2 to 24 hours. This indicates that it is a bactericidal agent against *E. faecalis*.

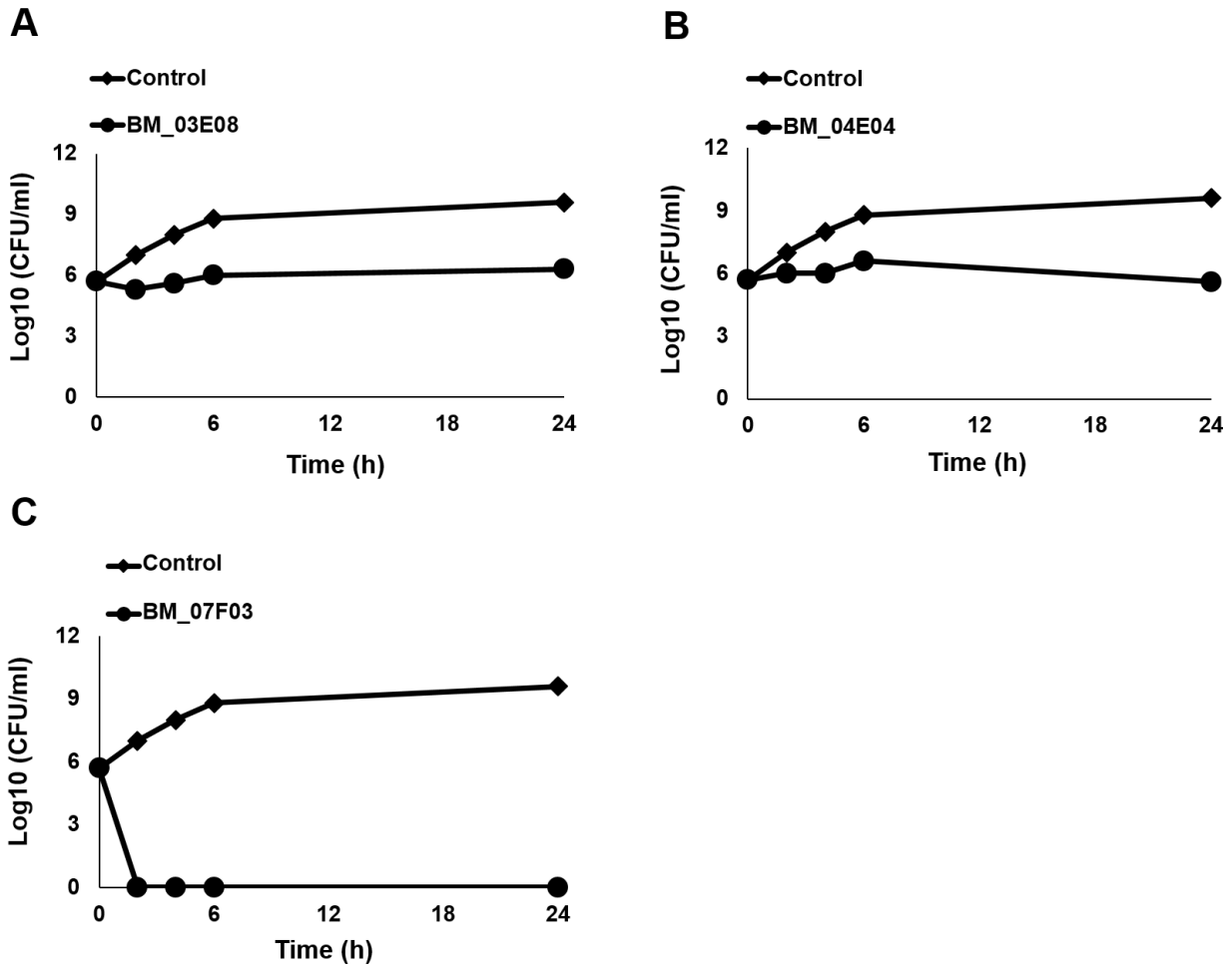


Fig 26. Time-kill kinetics of compound hits from library #2 against *E. faecalis*. Compound hits were added to bacterial cultures at four times the MIC. Samples were analyzed by plating and determining for live cell counts at 0, 2, 4, 6, and 24 h. (A) BM_03E08; (B) BM_04E04; (C) BM_07F03. Diamonds (♦) represent control cultures grown in the absence of inhibitor, and filled circles (●) represent cultures containing the tested compounds at 4×MIC.

Eukaryote protein synthesis assay with chemical compound library #2 hits. An ideal antibacterial compound would show potent inhibition against the bacterial target but little or no inhibition of the corresponding eukaryotic system.

Wheat germ extract assays. Wheat germ extract assays were used to determine whether the 3 chemical compounds inhibited cytoplasmic protein synthesis in eukaryotic systems. Poly(U) messenger RNA, yeast $tRNA^{Phe}$, [^{3}H]phenylalanine and Mg^{2+} concentrations were optimized for poly(Phe) synthesis in wheat germ extract assays. The results showed that none of the test compounds inhibited protein synthesis at concentrations up to 200 µM (Fig 27). The level of poly(Phe) synthesis in the wheat germ assays is only approximately 5% of that seen in the A/T assays, this is due to the limited number of ribosomes present in the wheat germ lysate.

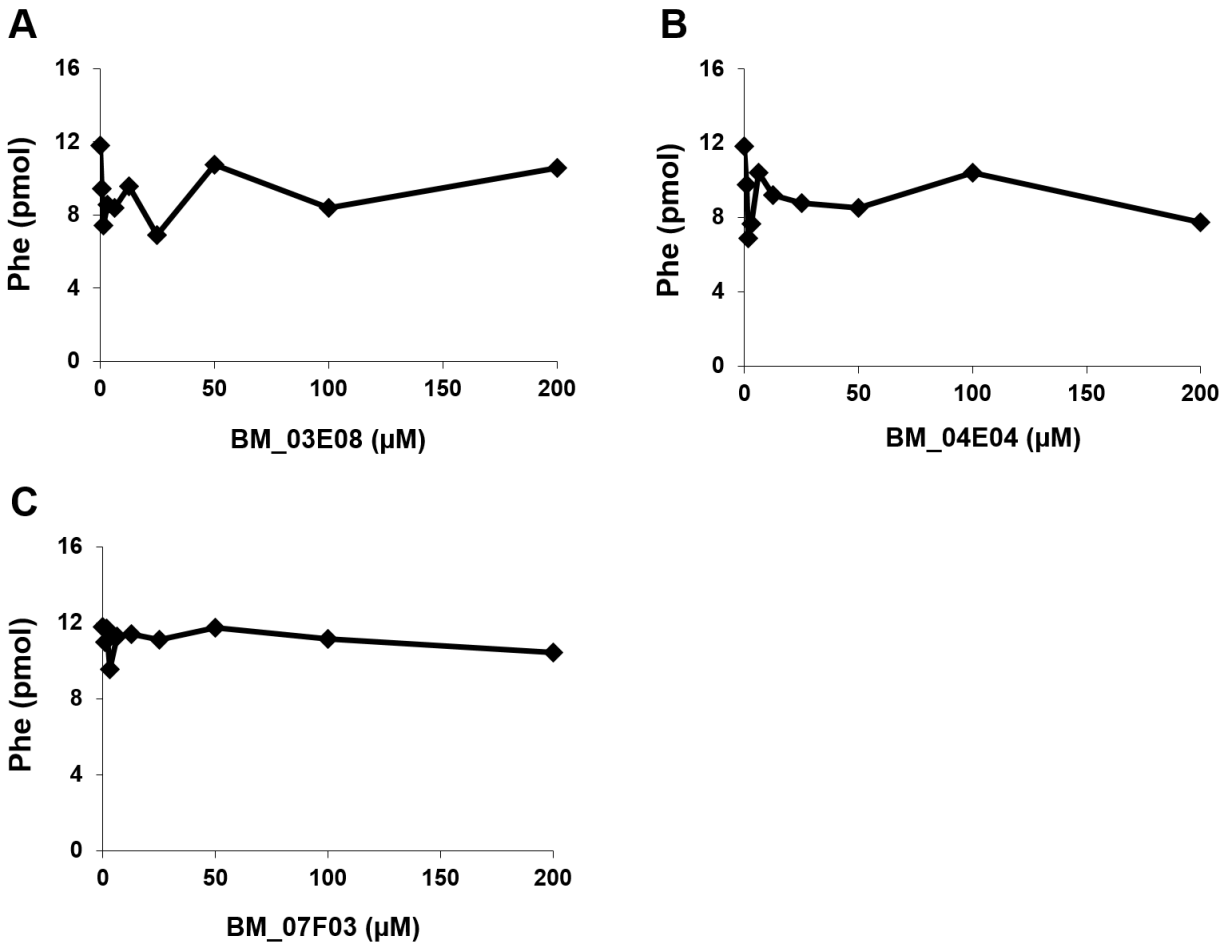


Fig 27. Wheat germ extract assays of chemical compounds. (A) BM_03E08; (B) BM_04E04; (C) BM_07F03.

Human mitochondrial PheRS assay. The human mitochondrial has its own protein synthesis system, and therefore inhibition of this system by the hit compounds is required to be monitored. BM_03E08, BM_04E04 and BM_07F03 inhibit *P. aeruginosa* PheRS, therefore inhibition of human mitochondrial PheRS by the 3 hit compounds was determined. The compounds were tested at concentrations up to 200 μM, and no inhibition was detected (Fig 28).

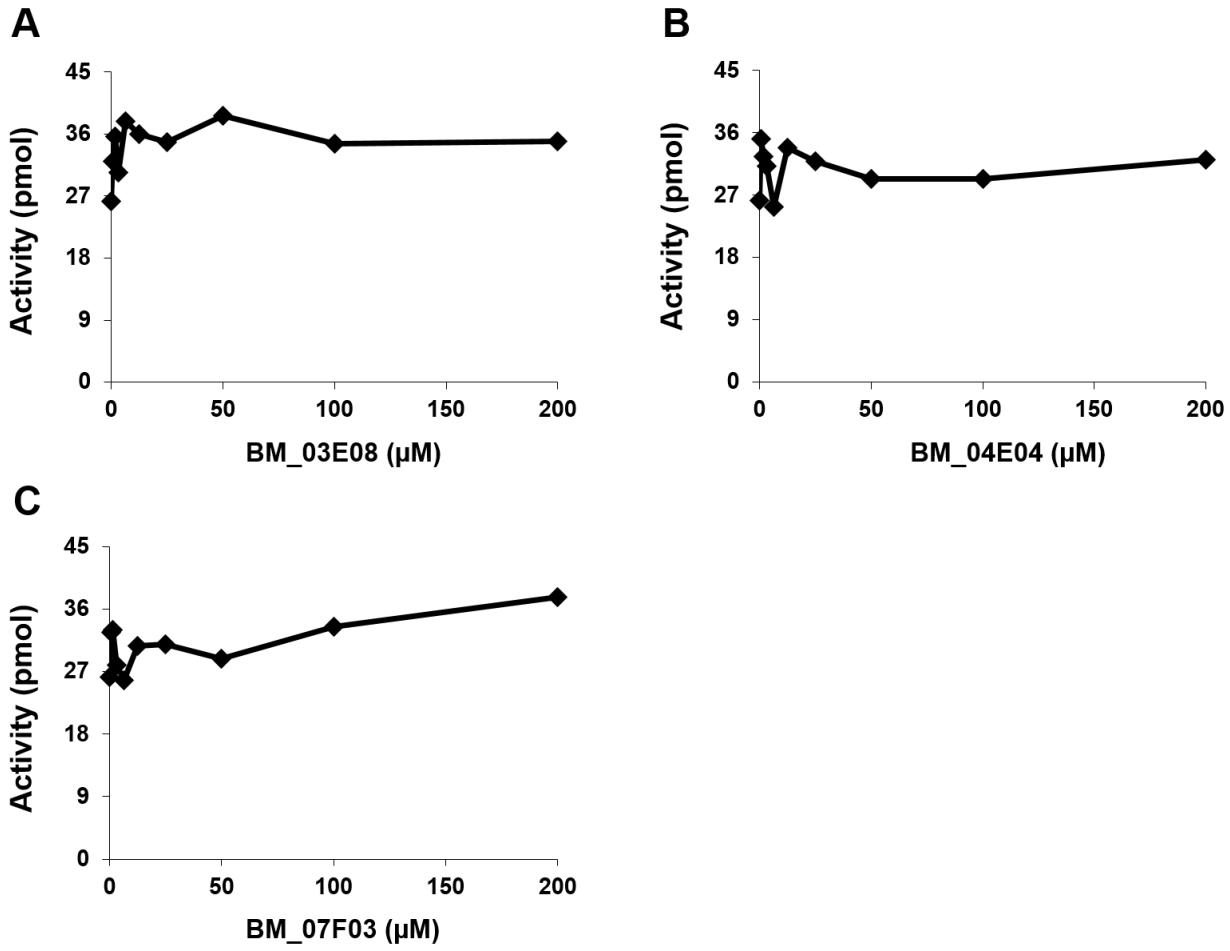


Fig 28. Human mitochondrial PheRS assay of chemical compounds. (A) BM_03E08; (B) BM_04E04; (C) BM_07F03.

Mode of action. The IC_{50}s for BM_03E08, BM_04E04 and BM_07F03 were determined at various concentration of ATP and Phe to determine the mode of action of the inhibitors. The mechanism of inhibition of the three compounds with respect to ATP was determined by the tRNA aminoacylation assay using various ATP concentrations (25, 50, 100, 250, 500, 1000 μM) ranging from 8-fold below to 5-fold above the K_m. The IC_{50}s for all three compounds increased with increasing ATP concentration (Fig 29), which is characteristic of a competitive inhibitor (66). To determine the mechanism of inhibition with respect to the amino acid, the same assay was used, except ATP was held constant at saturating concentration (2.5 mM) and the IC_{50} was determined at different concentrations of Phe (25, 50, 100, 200, 300 μM). The Phe concentrations ranged from approximately 1-fold below to 10-fold above the K_M. The IC_{50}s for BM_03E08 decreased with increasing Phe concentration (Fig 30A), which is characteristic of an uncompetitive inhibitor (66). The IC_{50}s for BM_04E04 increased with increasing Phe concentration (Fig 30B), which is characteristic of a competitive inhibitor (66). The IC_{50}s for BM_07F03 is constant with various concentrations of Phe (Fig 30C), which is characteristic of a noncompetitive inhibitor (66).

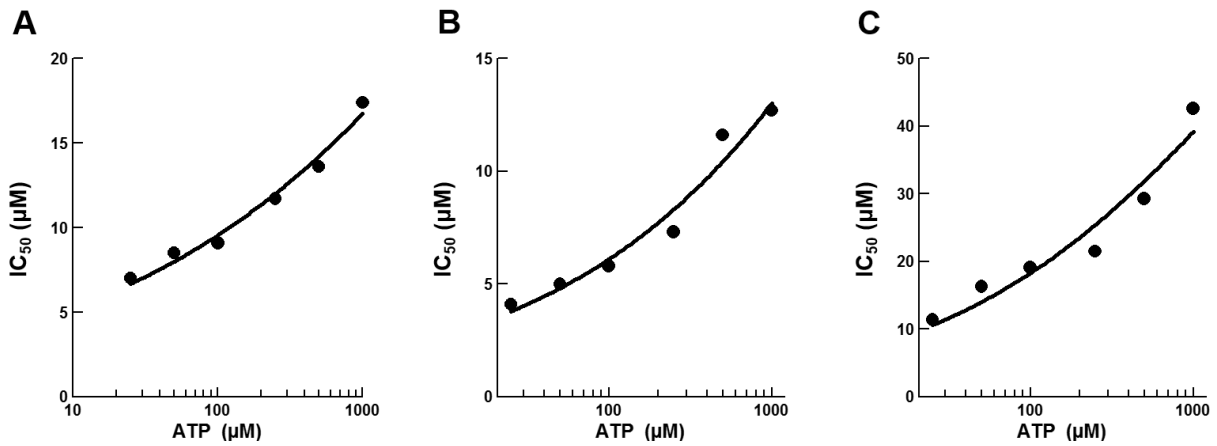


Fig 29. Determination of binding mode of the inhibitors relative to ATP. IC_{50}s for BM_03E08, BM_04E04 and BM_07F03 were determined using the aminoacylation assay. The phenylalanine concentration was fixed at 100 μM, and IC_{50}s were determined at six different ATP concentrations ranging from 25 to 1000 μM. The data was fit to the sigmoidal dose response model using XLfit (IDBS) using IC_{50}s determined in assays containing 0.1 μM *P. aeruginosa* PheRS. A) BM_03E08; B) BM_04E04; C) BM_07F03.

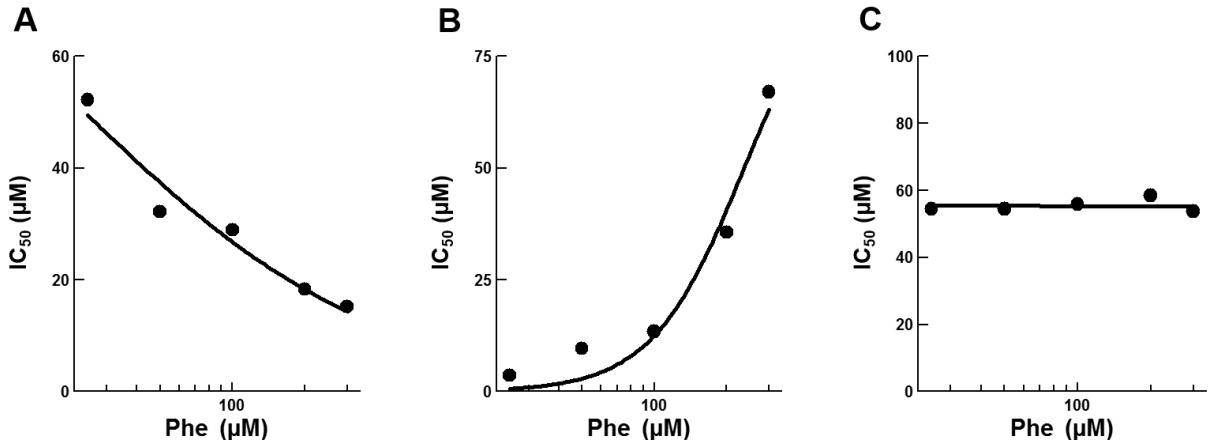


Fig 30. Determination of binding mode of the inhibitors relative to Phe. IC_{50}s for BM_03E08, BM_04E04 and BM_07F03 were determined using the aminoacylation assay. The ATP concentration was fixed at 2.5 mM, and IC_{50}s were determined at five different Phe concentrations ranging from 25 to 300 μM. The data was fit to the sigmoidal dose response model using XLfit (IDBS) using IC_{50}s determined in assays containing 0.1 μM *P. aeruginosa* PheRS. A) BM_03E08; B) BM_04E04; C) BM_07F03.

CHAPTER V

CONCLUSION

A protein synthesis system from *Pseudomonas aeruginosa* has been successfully developed and optimized with the concentrations of the enzymatic components set just below the inflection point of saturation on the titration curve to facilitate maximum sensitivity to inhibition, and the concentrations of non-enzymatic components set above the saturated point to avoid limitation. The A/T system was used to screen two chemical compound libraries, one compound library composed of 320 compounds (library #1), and the other one with 800 compounds (library #2).

From chemical compound library #1, 7 inhibitors were identified: 1 compound (BP_03B04) inhibited the function of PheRS, 2 compounds (BP_01C09, BP_02H03) inhibited EF-Tu, 4 compounds (BP_01F06, BP_02G06, BP_02G07 and BP_03C05) inhibited ribosomal function. MIC results showed that only three of the compounds had antibacterial activity: BP_01F06 inhibited growth of *M. catarrhalis* with an MIC value of 16 µg/ml and BP_01C09 displayed an MIC of 64 µg/ml; BP_02G07 inhibited growth of *S. aureus* with an MIC value of 64 µg/ml. The remaining compounds were not observed to inhibit any of the bacteria tested below the concentration of 128 µg/ml. BP_03B04, which was found to inhibit the function of *P. aeruginosa* PheRS, when assayed against cytoplasmic eukaryotic protein synthesis system from wheat germ extracts was shown not to inhibit activity of the system at the concentration up to 132 µM. However, when assayed against human mitochondrial PheRS up to 75% of the activity of this system was observed to be inhibited.

From chemical compound library #2, 13 inhibitors were identified: 1 compound (BM_03F11) inhibited the activity of EF-G, 5 compounds (BM_02F09, BM_03E08, BM_04E04, BM_07F03 and BM_10G05) inhibited PheRS, 7 compounds (BM_02H08, BM_03F03, BM04E05, BM05E06, BM_06G07, BM_09B09 and BM_10D05) inhibited the ribosomal activity.

MIC results showed that three of the inhibitors (BM_03E08, BM_04E04 and BM_07F03) have low MIC values against most of the pathogenic bacteria tested. Time kill study showed that of these three compounds, BM_07F03 was bactericidal against both *M. catarrhalis* and *E. faecalis*, while BM_03E08 and BM_04E04 both displayed bacteriostatic inhibition of both *M. catarrhalis* and *E. faecalis*. Wheat germ extract assays and human mitochondrial PheRS assays indicated that these three compounds displayed no inhibitory activity against eukaryotic cytoplasmic or mitochondrial protein synthesis. Therefore, these three compounds have a potential to be developed into new antibiotics.

Binding mode of BM_03E08, BM_04E04 and BM_07F03 have been studied. The results showed that BM_03E08 is competitive with respect to ATP and uncompetitive with respect to Phe; BM_04E04 is competitive with respect to both ATP and Phe; BM_07F03 is competitive with respect to ATP and noncompetitive with respect to Phe.

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APPENDIX A

APPENDIX A

MEDIUM AND AGAR COMPONENTS FOR BACTERIA IN MIC AND TIME KILL STUDY

Bacteria	**Medium**	**Agar**
M. catarrhalis	Brain Heart Infusion	Trypticase Soy Broth agar + 3% blood
E. faecalis	Brain Heart Infusion	Trypticase Soy Broth agar + 3% blood
S. pneumoniae	Brain Heart Infusion +3% blood	Trypticase Soy Broth agar + 3% blood
E. coli	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood
S. aureus	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood
H. influenzae	Haemophilus Test Medium	Chocolate Agar Plate
P. aeruginosa	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood
P. aeruginosa Hypersensitive strain	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood
P. aeruginosa PA200 Efflux	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood
E. coli TolC Efflux	Trypticase Soy Broth	Trypticase Soy Broth agar + 3% blood

APPENDIX B

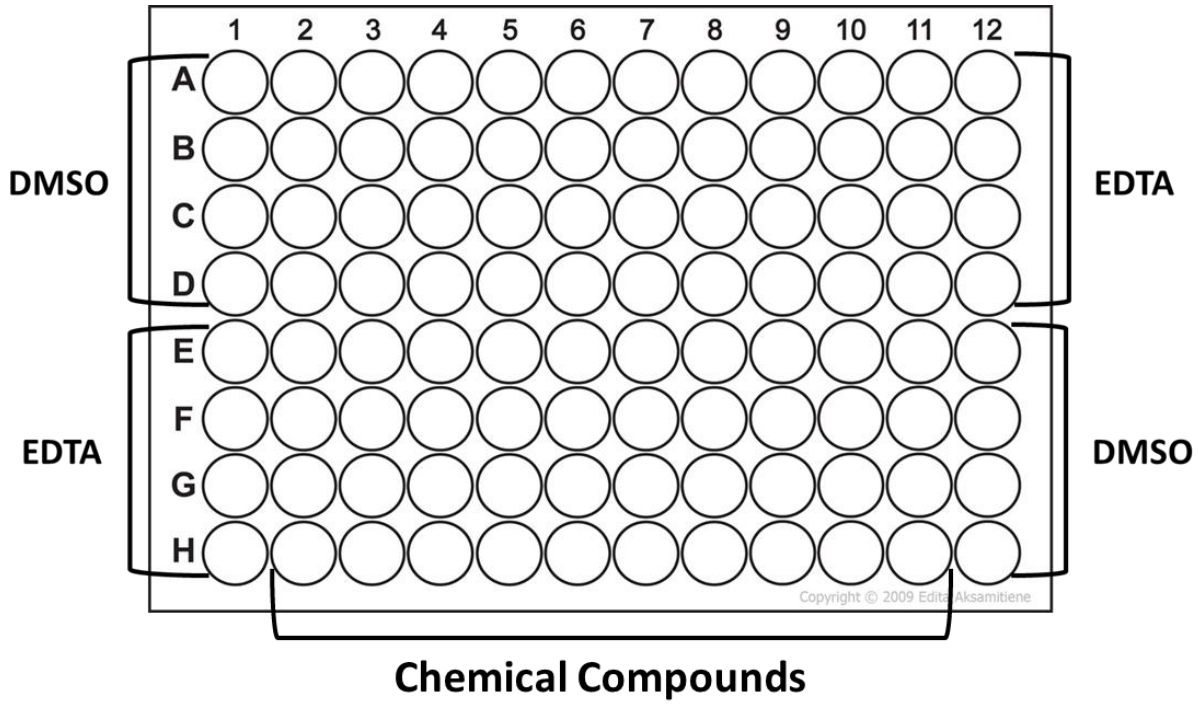
APPENDIX B

PROTOCOL FOR ALL ASSAYS USED IN THIS PROJECT

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

1) Dilute the parent plates of chemical compound library (10 mM) to the concentration of 3.3 mM with DMSO to make working plates.

2) Add 2 µl of DMSO, 0.5 M EDTA and 3.3 mM chemical compounds into 96 well microtiter plates (Costar) as indicated below:



3) Make master mix follow the recipe in the table below:

Master Mix	**Concentration**	**Stock**	**µl/Rx**
H_2O			16.54
Tris-HCl (pH 7.5)	50 mM	1 M	2.5
P. aeruginosa ribosomes	0.2 µM	11.6 µM	0.86
$MgCl_2$	10 mM	1M	0.5
KCl	25 mM	1M	1.25
PEP	4 mM	100 mM	2
PK	0.025 Units/µl	12.5 Units/µl	0.1
ATP	1.5 mM	100 mM	0.75
GTP	0.5 mM	100 mM	0.25
[^{3}H] Phenylalanine (75 cpm/pmol)	40 µM	1250 µM	1.6
Poly(U) mRNA	0.3 mg/ml	5 mg/ml	3
P. aeruginosa EF-Tu	1.0 µM	18.9 µM	2.7
P. aeruginosa EF-G	0.2 µM	50.1 µM	0.2
P. aeruginosa EF-Ts	0.05 µM	243 µM	0.01
Spermine	0.03 mM	10 mM	0.15
DTT	1 mM	1.0 M	0.05
P. aeruginosa PheRS	0.1 µM	7.04 µM	0.54
Total			33

4) Add 33 µl master mix to each well of the plate and incubate at room temperature for 15 min to allow thorough interaction of the enzymatic components with the compounds.

5) Add 15 µl *E. coli* tRNA solution (including 1.5 µl stock tRNA 670 µM and 13.5 µl H_2O, the concentration of total tRNA is 20 µM or 0.5 µM of tRNA-Phe) into each well of the plate to start the reaction.

6) Incubate the reaction at room temperature for 2 h, and stop it by adding 5 µl of 0.5 M EDTA.

7) Add 200 µg SPA beads (including148 µl 300 mM pH 6.2 Citrate buffer and 2 µl 100 mg/ml RNA binding beads [YSi]; Perkin-Elmer) to each well. Set in room temperature for at least 30 min.

8) Read the plates using a Perkin-Elmer scintillation counter.

2. IC_{50} assay

1) Dilute the chemical compound in a serial concentrations: 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625, 0.01953125 and 0.009765625 mM with DMSO.

2) Add 2 µl of the diluted compound into the 96-well plate, which makes the concentrations of the compound in the assay are 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.390625 µM.

3) Follow steps 3 to 8 in the initial screening assay.

3.3 Individual protein assay to determine target of compound hits

3. PheRS assay

1) Add 2 µl of 0.5 M EDTA (negative control), DMSO (positive control) and chemical compound hits into 96-well plate.

2) Make master mix follow the recipe in the table below:

Master Mix	**Concentration**	**Sstock**	**µl/Rx**
H_2O			24.51
Tris-HCl (pH 7.5)	50.0 mM	1.0 M	2.50
Spermine	1.0 mM	100 mM	0.5
$MgCl_2$	8 mM	1 M	0.40
ATP	2.5 mM	100 mM	1.25
Hot/Cold phe	50.0 µM	1.25 mM	3.00
P. aeruginosa PheRS	0.075 µM	7.04 µM	0.54
DTT	1 µM	1.0 M	0.05
Total			33

3) Add 33 µl master mix into the plate in 1), incubate in room temperature for 15 min, then start the reaction by adding 15 µl *E. coli* tRNA solution (including 6 µl stock tRNA 670 µM and 9 µl H_2O, the total tRNA concentration is 80 µM, or tRNA-Phe 2 µM) to each well. Incubate the reaction in 37 °C heat block for 1 h, and stop it by adding 5 µl 0.5 M EDTA.

4) Add 400 µg beads (including 146 µl 300 mM pH 2.0 Citrite buffer and 4 µl 100 mg/ml Ysi Poly-L-Lysine coated beads, Perkin-Elmer) to each well, set in room temperature for at least 1 hour.

5) Analyze the plates using a Perkin-Elmer scintillation counter.

4. LeuRS assay

1) Add 2 µl of 0.5 M EDTA (negative control), DMSO (positive control) and chemical compound hits into 96-well plate.

2) Make master mix follow the recipe in the table below:

Master Mix	**Concentration**	**Stock**	**µl/Rx**
H_2O			26.11
Tris-HCl (pH 7.5)	50.0 mM	1.0 M	2.50
MgOAc	5.0 mM	1 M	0.25
ATP	2.5 mM	100 mM	1.25
Hot/Cold Leu	50.0 µM	1.25 mM	2
P. aeruginosa LeuRS	0.1 µM	5.64 µM	0.89
Total			33

3) Add 33 µl master mix into the plate in 1), incubate in room temperature for 15 min, then start the reaction by adding 15 µl *E. coli* tRNA solution (including 6 µl stock tRNA 670 µM and 9 µl H_2O, the total tRNA concentration is 80 µM, or tRNA-Phe 2 µM) to each well. Incubate the reaction in 37 °C heat block for 1 h, and stop it by adding 5 µl 0.5 M EDTA.

4) Add 400 µg beads (including 146 µl 300 mM pH 2.0 Citrite buffer and 4 µl 100 µg/µl Ysi Poly-L-Lysine coated beads, Perkin-Elmer) to each well, set in room temperature for at least 1 hour.

5) Analyze the plates using a Perkin-Elmer scintillation counter.

5. EF-Tu assay

1) Add 2 µl DMSO or 3.3 mM Chemical Compound hits into glass tubes (on ice).

2) Make master mix follow the recipe in the table below:

Master Mix	Concentration	Stock	µl/Rx
H_2O			33.8
Tris-HCl (pH 7.5)	50 mM	1 M	2.5
NH_4Cl	50 mM	1 M	2.5
$MgCl_2$	10 mM	1 M	0.45
EF-Tu	1.0 M	18.9 µM	2.7
DTT	1.0 mM	1 M	0.05
Total			42

3) Add 42 µl master mix into the glass tubes in 1), incubate in room temperature for 15 min, then start the reaction by adding 6 µl 100 µM [^{3}H] GDP. The positive control contains 2 µl DMSO and 42 µl master mix, the negative control contains 2 µl DMSO and 42 µl master mix without EF-Tu, and the 2.7 µl EF-Tu is substituted by 2.7 µl H_2O.

4) Incubate the reactions for 30 min in 37 °C water bath.

5) Stop the reaction by adding 1.0 ml of cold wash buffer (50 mM Tris (pH 7.5), 50 mM NH_4Cl, 10 mM $MgCl_2$), and place the glass tubes on ice.

6) Filter through the reaction solution onto Nitro-cellulose filters, which was previously soaked in 5% TCA. Wash the filters 3 times with 2 ml of wash buffer. Filters are dried and counted on a Perkin-Elmer scintillation counter.

6. EF-Ts assay

1) Add 2 µl DMSO or 3.3 mM Chemical Compound hits into glass tubes (on ice).

2) Make master mix follow the recipe in the table below:

Master Mix	Concentration	Stock	µl/Rx
H_2O			32.55
Tris-HCl (pH 7.5)	50 mM	1 M	2.5
NH_4Cl	50 mM	1 M	2.5
$MgCl_2$	10 mM	1 M	0.45
EF-Tu	1.0 µM	19.2 µM	2.8
DTT	1.0 mM	500 mM	0.1
EF-Ts	0.05 µM	2.43 µM	1.1
Total			42

3) Add 42 µl master mix into the glass tubes in 1), incubate in room temperature for 15 min, then start the reaction by adding 6 µl 100 µM [^{3}H] GDP. The positive control contains 2 µl DMSO and 42 µl master mix, the negative control contains 2 µl DMSO and 42 µl master mix without EF-Ts, and the 1.1 µl EF-Ts is substituted by 1.1 µl H_2O.

4) Incubate the reactions for 30 s at 37 °C water bath.

5) Stop the reaction by adding 1.0 ml of cold wash buffer (50 mM Tris (pH 7.5), 50 mM NH_4Cl, 10 mM $MgCl_2$) and then place the glass tubes on ice.

6) Filter through the reaction solution onto Nitro-cellulose filters, which was previously soaked in 5% TCA. Wash the filters 3 times with 2 ml of wash buffer. Filters are dried and counted on a Perkin-Elmer scintillation counter.

6. EF-G assay

1) Add 2 µl of 0.5 M EDTA (negative control), DMSO (positive control) and 3.3 mM chemical compound hits into 96-well plate.

2) Make master mix follow the recipe in the table below:

Master Mix	**Concentration**	**Stock**	**µl in assay**
H_2O			30.55
Tris-HCl (pH 7.5)	50.0 mM	1.0 M	2.50
$MgCl_2$	10.0 mM	1 M	0.50
NH_4Cl	70 mM	1 M	3.50
EF-G	0.2 µM	50.1 µM	0.2
P. seudomonas ribosomes	0.4 µM	11.6 µM	1.70
DTT	1 mM	1 M	0.05
Total			39

3) Add 39 µl master mix into each well in the plate in 1), incubate in room temperature for 15 min, then start the reaction by adding 9 µl 10 mM GTP.

4) Incubate the reaction at 37 °C heat block for 30 min.

5) Stop the reaction by adding150 µl of 50 mM EDTA.

6) Add 50.5 µl Gold Lock Mix (including 50 µl Gold Lock and 0.5 µl Accelerator) to each well, set in room temperature for 10 min.

7) Read absorbance at 595 nm using the Multiscan software.

7. MIC assay

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

2) Prewarm 15 ml broth growth medium to room temperature.

3) Pick 3-5 colonies on the agar plate, inoculate into the medium of step 2. 35 °C, 200 rpm grow 2 to 6 h or until the OD_{625} reaches 0.08 to 0.13.

4) Dilute the bacteria in step 3 to OD_{625} value in the range of 0.08 to 0.13 if needed.

5) Prepare microtiter plate: Add 90 µl of growth medium of step 4 into wells of column 1 and 50 µl of medium into wells 2 through 12.

6) Add 10 µl of compound needed to do MIC test (1.28 mg/ml) into the first column. The last row of each plate is to contain the QC agent.

7) Serially dilute compound by transferring 50 µl medium from column 1 through column 11 with thorough mixing. Discard 50 µl from column 11.

8) Incubate plates at 37 °C.

9) Read MIC results after incubation for 18-24 h.

8. Time-kill study

1) Overnight grow bacteria the day prior to the time kill study.

2) Inoculate 10 ml of medium with 0.1 ml of fresh overnight sample in step 1, 35 °C 200 rpm grow for 2 h or until its OD_{625} above 0.08-0.13.

3) Prepare 2 shake flasks with 10 ml of the following:

Medium along (control);

Medium +chemical compound at 4 × MIC.

Prewarm the flasks to room temperature.

4) Prepare microtiter plates for sample dilution: Add 90 µl of 0.85% sterile saline solution to column 2-8. Leave column 1 empty for sample addition.

5) Dilute sub-culture in step 2 to OD_{625} in the range of 0.08 to 0.13 by adding culture to 5 ml 0.85% sterile saline solution until match.

6) Inoculate 0.1 ml of the sub-culture in step 5 into the flasks of step 3.

7) Immediately following inoculation, transfer 100 µl samples of the flasks into first well in one of the microtiter plates in step 4, label as 0 h.

8) Start timer and incubate culture in step 6 at 35 °C, 200 rpm.

9) Serially dilute the 0 h sample as follows: transfer 10 µl of sample from column 1 to column 2 and mix well, repeat these 10-fold serial dilutions up to column 8.

10) Working row by row, spot 10 µl of each sample dilution onto an agar plate subdivided into eight zones. From highest concentration to lowest concentration: 10E02, 10E03, 10E04, 10E05, 10E06, 10E07, 10E08, 10E09.

11) Let plates air dry until spots have soaked in, and incubate plates at 37 °C.

12) Repeat sampling at 2, 4, 6 and 24 h.

13) Enumerate colonies the following day and calculate CFU/ml (multiply by 10 × number of serial dilution × 10E+02).

9. Wheat germ extract assay

1) Dilute the chemical compound that needed to do wheat germ assay into 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625 and 0.01953125 mM with DMSO.

2) Add 2 µl of the diluted compound into the glass tubes, which makes the concentrations of the compound in the assay are 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 µM.

3) Make master mix follow the recipe in the table below:

Master Mix	Concentration	Stock	µl/Rx
H_2O			10.7
poly (U) RNA	3 mg/ml	5 mg/ml	3
3H Phenylalanine (100 cpm/pmol)	75 µM	1250 µM	3
Wheat Germ Extract			30
Yeast $tRNA^{Phe}$	4 µM	200 µM	1
MgOAc	6 mM	1 M	0.3
Total			48

4) Add 48 µl master mix to each glass tube and incubate at room temperature for 2 hours.

5) Stop the reaction by adding 2 ml of 5% TCA.

6) Place the reaction tube in a 90 °C block heater for 10-15 minutes.

7) Vacumn filter the reaction solution onto Whatman glass-fiber filters which was previously soaked in 5% TCA.

8) Wash the filters with 5 ml of 5% TCA twice.

9) The filters were dried and counted in a scintillation counter.

10. Human mitochondrial PheRS assay

1) Dilute the chemical compound that needed to do human mitochondrial assay into 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625 and 0.01953125 mM with DMSO.

2) Add 2 µl of the diluted compounds into the glass tubes, which makes the concentrations of the compound in the assay are 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 µM.

3) Make master mix follow the recipe in the table below:

Master Mix	**Concentration**	**Stock**	**µl/Rx**
H_2O			24.5
Tris-HCl (pH 7.5)	50.0 mM	1.0 M	2.5
Spermine	1.0 mM	100 mM	0.5
$MgCl_2$	8 mM	1 M	0.4
ATP	2.5 mM	100 mM	1.25
Hot/Cold phe	50.0 µM	1.25 mM	3
hmPheRS	0.5 µM	46 µM	0.55
DTT	1 µM	1.0 M	0.05
Total			33

4) Add 33 µl master mix to each glass tube in 1) and incubate in room temperature for 15 min, then start the reaction by adding 15 µl *E. coli* tRNA solution (including 7.6 µl stock tRNA 528 µM and 7.4 µl H_2O, the total tRNA concentration is 80 µM, or tRNA-Phe 2 µM) to each tube. Incubate the reaction in 37 °C water bath for 1 hour.

5) Stop the reaction by adding 2 ml of 5% TCA.

6) Vacumn filter the reaction solution onto Whatman glass-fiber filters which was previously soaked in 5% TCA.

7) Wash the filters with 5 ml of 5% TCA twice.

8) The filters were dried and counted in a scintillation counter.

BIOGRAPHICAL SKETCH

The author, Yanmei Hu (ynhu@broncs.utpa.edu or ymhu1982@gmail.com), was born on February 14th, 1982 in China.

She earned her Bachelor of Engineering degree in Applied Chemistry from Yangtze University in 2004. She received her Master of Science degree in Cell Biology from Hebei Normal University in 2008. In 2012, she joined the graduate program in Chemistry at the University of Texas Pan-American.