Identification of Novel Lead Inhibitors for Bacillus anthracis Adenylosuccinate Synthetase

BY

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Thesis

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This thesis is dedicated to my parents, without whom it would never have been accomplished.

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTS	High throughput screening
IMP	Inosine monophosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_m	Michaelis constant
LB	Luria broth
MESG	2-amino-6-mercapto-7-methylpurine riboside
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
OD	Optical density
OM	Outer membrane
PBP	Pencillin binding protein

LIST OF ABBREVIATIONS (continued)

PNP	Purine nucleoside phosphorylase
P_i	Inorganic phosphate
PurA	Adenylosuccinate synthetase
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Terrific broth
TCEP	Tris(2-chloroethyl) phosphate

SUMMARY

Anthrax is an acute infectious disease caused by the spore-forming bacterium *Bacillus anthracis.* Anthrax is a serious disease that came into public notability in 2001 during the bioterrorism attack in the United States. It is classified as a Category A agent – the highest risk type - by the Centers for Disease Control and Prevention (CDC). The standard treatment for anthrax is a 60-day course of an antibiotic, such as tetracycline, ciprofloxacin or doxycycline. However, it is easy to select *B. anthracis* strains that are resistant to various classes of antibiotics including penicillin, erythromycin, and trimethoprim. In addition, there may be biologically mutant strains that are engineered to be resistant to various antibiotics. There is currently a vaccine against the anthrax causing bacterium but the main problem with the vaccine is that it is given as multiple doses over a period of 18 months, so it is unlikely to provide safety to the people in the case there is an outbreak of bacterial attack. Thus, it is necessary to develop novel therapeutic agents against *B. anthracis*.

The long-term objective of my research is to develop novel antibacterial agents against adenylosuccinate synthetase (PurA) from *B. anthracis*. Adenylosuccinate synthetase is an important enzyme in the bacterial purine biosynthesis pathway. PurA catalyzes the first committed step in the *de novo* biosynthesis of AMP, coupling the hydrolysis of GTP to the formation of adenylosuccinate from aspartate and IMP. Various knockout studies have shown that PurA is essential for survival of bacteria. Inhibitors designed against PurA might provide us with a broad-spectrum antibiotic.

To discover lead compounds that will inhibit PurA from *B anthracis*, various strategies were implemented which includes protein expression and purification, assay optimization for high-

throughput screening, use of different chemical libraries (e.g. Life Chemicals, Chembridge) for the high throughput screening (HTS). The primary hits were analyzed and validated using tryptophan fluorescence assay.

CHAPTER 1: Literature Review

1.1 Anthrax : A threat

A biological attack, or bioterrorism, is the intentional release of viruses, bacteria, or other germs that can sicken or kill people, livestock, or crops. The first intended use of anthrax as an act of warfare was recorded during World War I [1, 2].

German army used anthrax to infect livestock and animal feed traded to the Allied Nations by neutral partners. An example of this act of aggression was the infection of Argentinian livestock that resulted in the death of 200 mules in 1917 and 1918 [1, 2]

Japan began using anthrax as a weapon from 1932 until the end of World War II. The research and development was conducted in military units located at Manchuria, which was occupied by Japan. During this time, prisoners were infected with anthrax and other deadly diseases. It was later discovered that during this program, more than 10,000 prisoners were believed to have died as a result of experimental infection [3, 4]. During 1979, an epidemic of anthrax was reported among people in the city of Sverdlovsk, USSR [5].

Bioterrorist attacks in the U.S occurred between October and November 2001, when *B.anthracis* spores were sealed in envelopes and sent to government officials, and news media industries through US postal service. At least twenty-two people developed anthrax infections, with eleven of these in the especially life-threatening inhalational variety. Five of them died [6]. Anthrax has been rated as Category A priority pathogen (NIH/NIAID) and also rated second in potential bioterrorism impact behind only smallpox [7].

1.2. An introduction to *Bacillus anthracis* and the disease anthrax

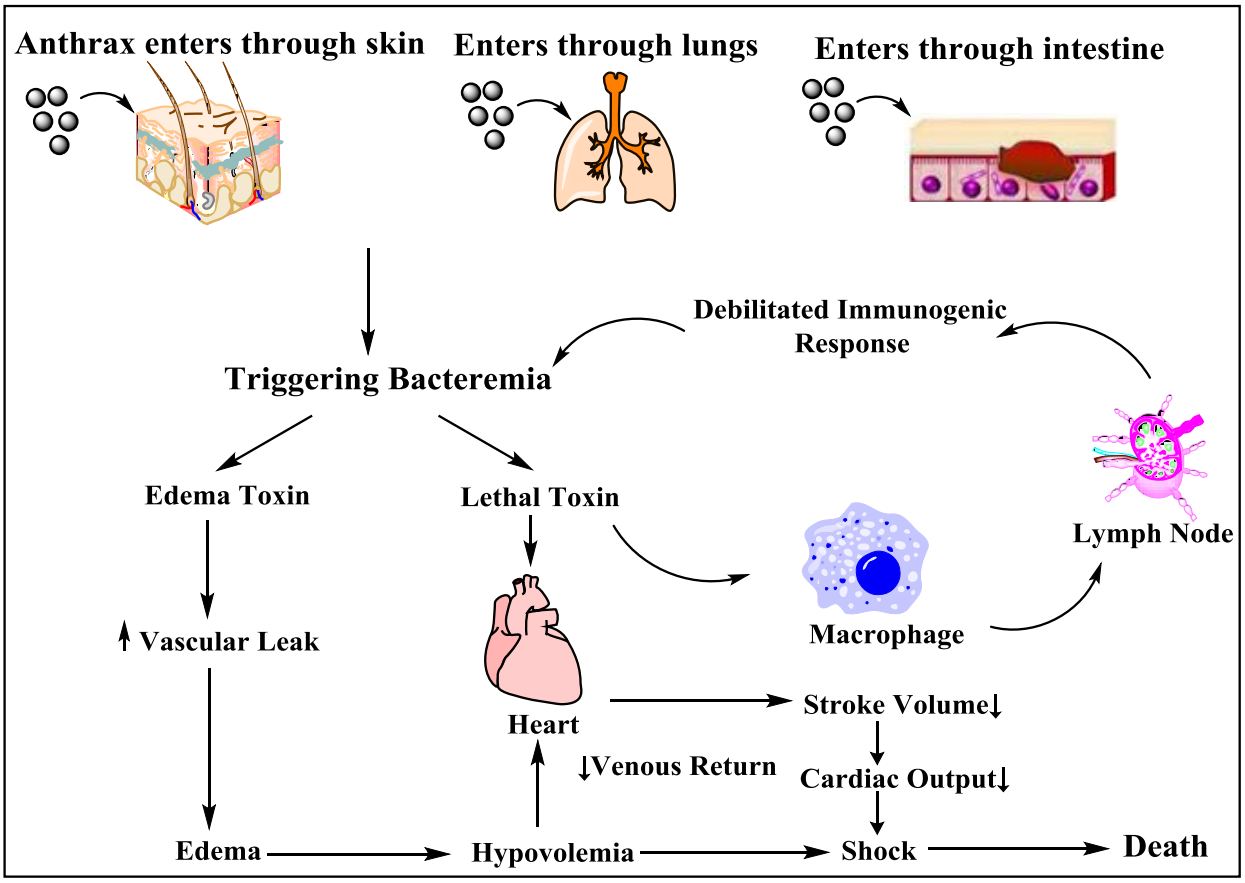


Figure 1.Pathophysiology of Anthrax (adapted from Krok 2. Medicine- 2009)

Anthrax is a serious infectious disease caused by gram-positive, rod-shaped bacteria known as *Bacillus anthracis*. The *B. anthracis* spores can survive in a dormant state in the environment, usually for several years. These spores are activated, and the bacteria begin to reproduce when inhaled in the body. Two toxins known as lethal toxin and edema toxin are released by this reproducing bacteria. The toxins cause a fatal buildup of fluid around the lungs that can kill infected cells and cause death (Figure 1).

Anthrax is believed to be threatening because it is highly lethal, one of the easiest biological agents to manufacture and develop as a weapon, easily spread in the air over a large area, and easily stored for several years.

Three forms of anthrax infection can occur: cutaneous (skin), inhalation, and gastrointestinal [8].

Cutaneous: "Most (about 95%) anthrax infections occur when the bacterium enters a cut or abrasion on the skin, such as when handling contaminated wool, hides, leather or hair products (especially goat hair) of infected animals. About 20% of untreated cases of cutaneous anthrax will result in death. Deaths are rare with appropriate antibacterial therapy" [8].

Inhalation: Inhalation anthrax is usually fatal. Initial symptoms may resemble common cold.

Intestinal: "The intestinal disease form of anthrax may be due to the consumption of contaminated meat and is characterized by an acute inflammation of the intestinal tract. Intestinal anthrax results in death in 25% to 60% of cases" [8].

An anthrax bacterial spore can withstand extreme conditions such as high and low temperature, drought, no nutrients, and even no air. When environmental conditions are favorable, the spores will germinate into flourishing colonies of bacteria.

"While spore formation allows the bacteria to survive in any environment, the ability to produce toxins makes the *B anthracis* such a potent killer. Both, the tolerance and toxicity of *B. anthracis* make it a formidable bioterror agent. Its toxin is made of three proteins: protective antigen, edema factor, and lethal factor" [9, 10]. "Protective antigen binds to select cells of an infected person or animal and forms a channel that permits edema factor and lethal factor to enter those cells. Edema factor, once inside the cell, causes fluids to accumulate at the site of infection.

Edema factor can contribute to a fatal build-up of fluid in the cavity surrounding the lungs." Lethal factor also works inside the cell and disrupts a key molecular switch that regulates the cell's functions. Lethal factor can kill infected cells or prevent them from working properly (Figure 2).

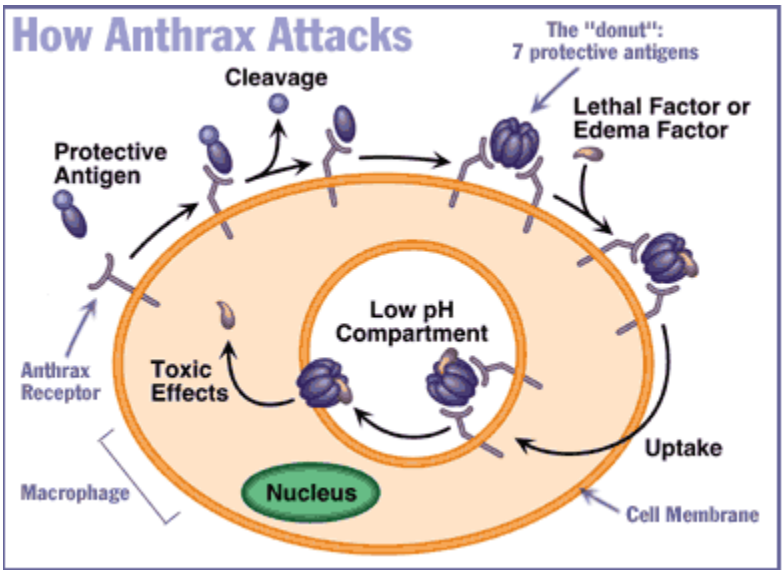


Figure 2.Mechanism of attack by *B. anthracis* (reproduced with permission from whyfiles.org)

1.3. Current treatment

Anthrax can only be treated if it is detected at early stage. Unfortunately, early symptoms of anthrax are often confused with symptoms of common infections and people do not seek medical advice until severe symptoms appear. By that time, the anthrax toxins have already risen to excessive levels, making therapy difficult. Antibiotics can be bactericidal, but antibiotics have no effect on anthrax toxins. Various antibiotics such as penicillin, tetracycline, and fluoroquinolones can be used and are effective if they are given before the onset of lymphatic spread, estimated to be 24 hours. The Food and Drug Administration approved an anthrax vaccine in 1970's which is licensed only to protect military personnel and people most at the risk for exposure to the

bacteria like people who work on farms and work with livestock. It is not recommended for public to take the vaccine as anthrax illness does not occur often and the vaccine may cause unfavorable effects in some people because its safety and efficacy have not been determined yet for children, the elderly, and people with weak immune systems.

Also the main antibiotics approved by the Food and Drug Administration for treatments of inhalational anthrax are doxycycline and ciprofloxacin [11]. However, it is fairly easy to select *B. anthracis* to be resistant to various classes of antibiotics, including β-lactams such as penicillin G, amoxicillin, erythromycin, cefuroxime, sulfamethoxazole, trimethoprim, aztreonam and ceftazidime [12-16]. Moreover, it was reported that selection for resistance to rifampin [17], ofloxacin [18], and tetracycline and penicillin classes [19]. Thus, there is clear evidence that drug-resistant strains can be isolated without great difficulty. In response to this, the development of new therapeutic agents against *B. anthracis* is very important.

1.4. Purpose and significance of study

1.4.1 The antibiotic resistance problem

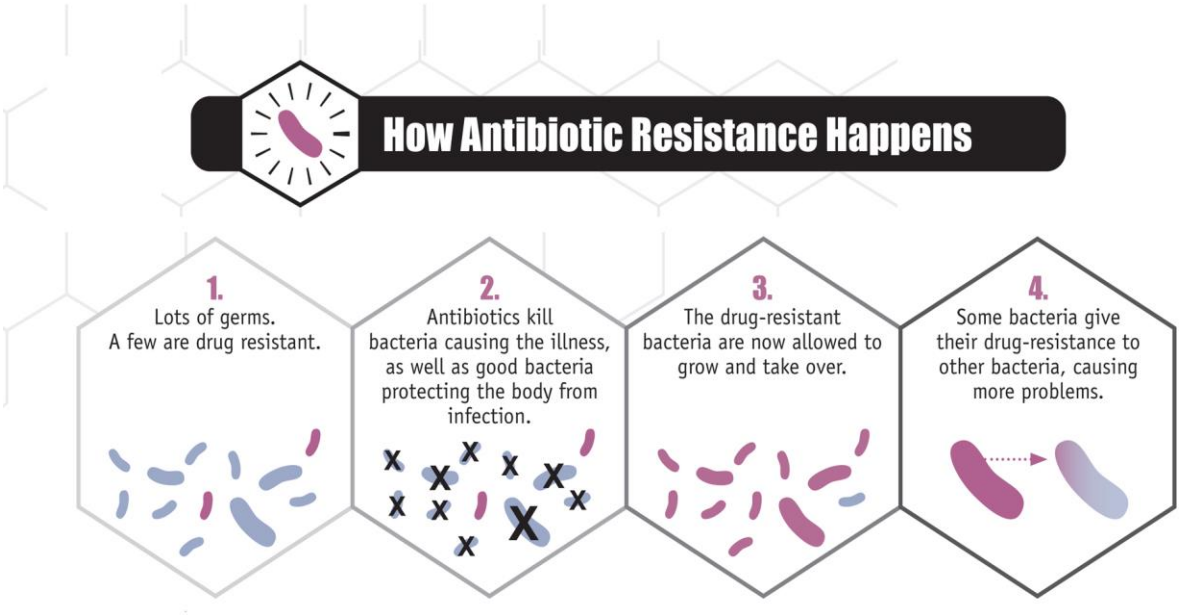
Antibiotic resistance is a growing medical problem. Antibiotic resistance has become a serious situation and has emerged as one of the leading public health concerns of the 21st century.

Antibiotic resistance has become an inevitable consequence of antibiotic overuse and is now prevalent is many bacterial pathogens. Often the antibiotic resistance genes originate in microorganisms that produce antibiotics. Use of antibiotics can increase selective pressure in bacteria to allow the resistant bacteria to flourish and the susceptible bacteria to die off.

However, bacteria may also have multiple intrinsic mechanisms that can make them less susceptible to antibiotics. The relevant mechanisms may include production of enzymes that inactivate the drug, alteration of the drug target, expression of efflux pumps that forces out antibiotics from the cell, use of an alternative biochemical pathway and more.

Now, clinically important bacteria are being discovered not only by resistance to single antibiotic, but also resistance to multiple antibiotics. Earlier reports of resistance among bacteria include- *Escherichia coli*, Shigella and Salmonella, to multiple drugs were made in the late 1950s to early 1960 [20, 21]. Now, the most important resistant microorganisms are currently methicillin-(oxacillin-)-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci [22, 23]. Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria are resistant to all beta-lactam antibiotics such as methicillin, penicillin, oxacillin, and amoxicillin. According to the Centers for Disease Control and Prevention (CDC), MRSA infections resulted in ~19,000 deaths in USA in 2012. In addition, resistant species which were once a problem only in the hospital setting, such as MRSA, are now increasingly prevalent in the wider community as well [24]. *Bacillus anthracis*, a notorious bacterium that was created as bioweapon back in World War I, still acts as a latent threat to public health. The emergence of antibiotic resistance *B anthracis* has also been reported [12].

As antibiotics resistance has becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decrease in the number of newly approved antibiotics. Antibiotic resistance therefore poses a significant problem (Figure 3).



CDC/ Melissa Brower

Figure 3.Mechanism of antibiotic resistance (http://www.cdc.gov/drugresistance)

1.4.2. Targets of currently available antibiotics

1.4.2.1. Mechanism of antibiotic action

The majority of the antibiotics generally fall within one the following categories: - cell wall synthesis inhibition, folate synthesis inhibition, fatty acid synthesis inhibition, protein synthesis inhibition, nucleic acid synthesis inhibition (Figure 4).

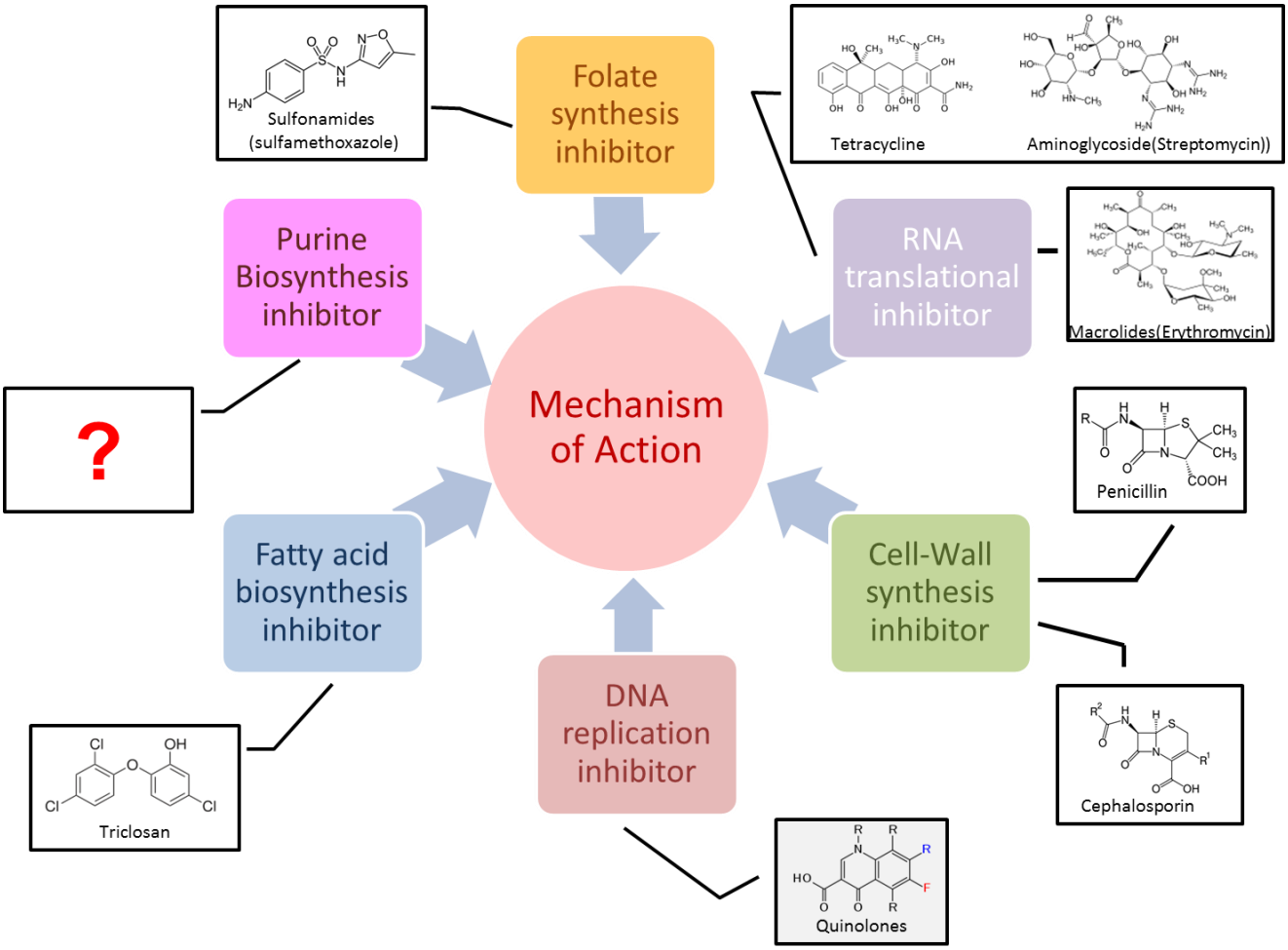


Figure 4. Mechanism of action of current antibiotics

Bacteria resemble primitive plants in that the cellular contents are surrounded by an inner peptidoglycan cell wall in addition to an inner plasma membrane and, in Gram-negative bacteria, an outer lipid bilayer. Specific anti-bacterial interferes with the synthesis of the cell wall, weakening the peptidoglycan scaffold within the bacterial wall so that the structural integrity eventually fails. Since mammalian cells have a plasma membrane but lack the peptidoglycan wall structure, this class of anti-bacterial selectively targets the bacteria with no significant negative effect on the cells of the mammalian host. Antibiotics that disrupt the synthesis of the

cell wall include the β-lactams, which bind to the penicillin-binding proteins (PBP). The β-lactams consist of β-lactam ring that is necessary for its activity. These drugs represent 60 % of all antimicrobial use by weight and include penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems [25]. The glycopeptides, such as vancomycin, inhibit the synthesis of the cell wall differently from the β-lactams: they sequester the peptide substrate and therefore prevent their crosslinking by the PBP's. Vancomycin is often considered as the last resort when all other antibiotic treatments have failed, especially for MRSA. The peptidoglycan is weakened which leads to lysis of the bacteria by use of β-lactams antibiotics.

Another class of antibiotic is one that inhibits DNA synthesis. Antibiotics such as quinolones interfere with DNA synthesis by inhibiting enzyme topoisomerase. Generally it is topoisomerase II (DNA gyrase), an enzyme involved in DNA replication which is inhibited. DNA gyrase relaxes supercoiled DNA molecules, and rejoins phosphodiester bonds in superhelical turns of closed-circular DNA. DNA or RNA polymerases then replicate the DNA strand. The second-generation quinolones, fluoroquinolones include antibiotics such as levofloxacin, norfloxacin, and ciprofloxacin. These are active against both Gram-positive and Gram-negative bacteria [26] .

A third class of antibiotic is the protein synthesis inhibitors. "Protein synthesis is a multi-step process that involves many enzymes as well as many substrates. However, most of antibiotics acts at the ribosome level and interfere with the processes at the 30S subunit or 50S subunit of the 70S bacterial ribosome (Figure 5). Each amino acid required for peptide synthesis is activated by the aminoacyl-tRNA synthetase.This synthetase, however, is not an antibiotic target. Instead, the steps on which antibiotics acts are (1) the formation of the 30S initiation complex (made up of mRNA, the 30S ribosomal subunit, and formyl-methionyl-transfer RNA), (2) the

formation of the 70S ribosome by the 30S initiation complex and the 50S ribosome, and (3) the elongation process of gathering amino acids into a polypeptide. Tetracyclines, including doxycycline, blocks the A (aminoacyl) site of the 30S ribosome and prevents the binding of aminoacyl-tRNA to the ribosome. Tetracyclines can inhibit protein synthesis in both 70S and 80S ribosomes, but they have preference to bind to bacterial ribosomes due to structural differences in RNA subunits. Streptomycin, one of the most commonly used aminoglycosides, interferes with the creation of the 30S initiation complex. Erythromycin, a macrolide, interferes with the assembly of 50S subunits by binding to the 23S rRNA component of the 50S ribosome. It blocks the 50S polypeptide export tunnel that prevents elongation at the transpeptidation step of synthesis". Chloramphenicol inhibits the attachment of mRNA to the 50s ribosome subunit [27].

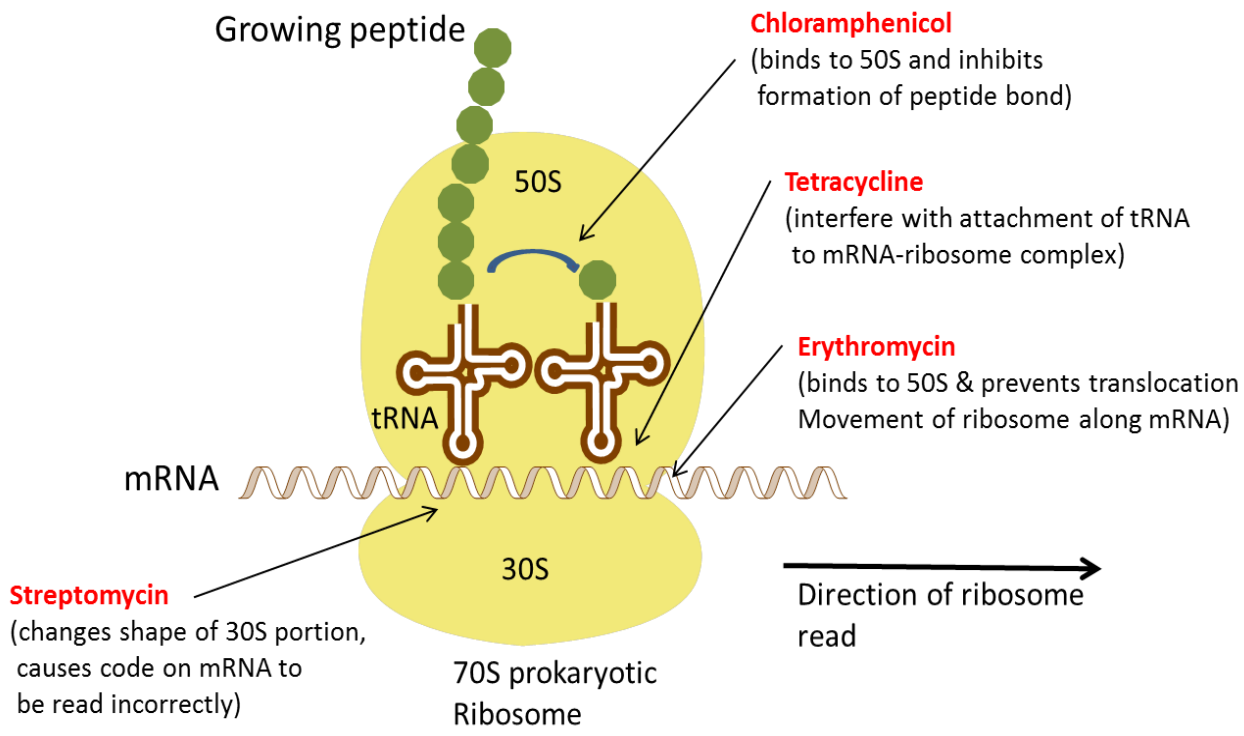


Figure 5.Protein Synthesis inhibition by antibiotics (adapted from book "Microbiology: An Introduction")

1.4.2.2. Mechanism of antibiotic resistance

Antibiotic resistance is a form of drug resistance whereby some sub-populations of a microorganism, usually a bacterial species, are able to survive after exposure to one or more antibiotics. Several mechanisms have evolved in bacteria which confer them with antibiotic resistance [28]. One of these mechanisms is decreased penetration of antibiotic to the target site. The outer membrane of gram-negative bacilli provides an efficient barrier to the penetration of β-lactam antibiotics to their target PBPs in the bacterial plasma membrane. Beta-lactams usually must pass through the hydrophilic porin protein channels in the outer membrane of gram-negative bacilli to reach the periplasmic space and plasma membrane. Another bacterial defense

against the action of the antibiotics is the alteration of the target site of antibiotics. The target sites for the beta-lactams are the PBPs in the cytoplasmic membrane. Alterations in PBPs may influence their binding affinity for beta-lactam antibiotics and therefore the sensitivity of the altered bacterial cell to inhibition by these antibiotics. Such a mechanism is responsible for penicillin resistance in pneumococci [29], and methicillin (oxacillin) resistance in staphylococci [30]. Another common mode is inactivation of antibiotic by a bacterial enzyme. Production of beta-lactamase is a major mechanism of resistance to the beta-lactam antibiotics in clinical isolates. Such bacterial enzymes may cleave predominantly penicillins (penicillinases), cephalosporins (cephalosporinases), or both (beta-lactamases). Their production may be encoded within the bacterial chromosome (and hence be characteristic of an entire species) or the genes may be acquired on a plasmid or transposon (and hence be characteristic of an individual strain rather than the species). An alternative strategy used by bacteria is the alteration in the charge or structure of the outer membrane polysaccharides which decreases the binding of certain antibiotics or loss of expression porins, or channels, to limit the entry of antibiotics [31]. In order to enter the cell, the antibiotic must pass the permeability barriers of the bacteria- the cell wall and the cytoplasmic membrane (CM) and the outer membrane in the case of the gram-negative bacteria. Transporters and porins are present on the outer membrane of the bacteria which allows intake of nutrients and ions, extravasation of metabolites, and response to the environment. Several studies have proven that changes in the structure of OM are associated with an increase in resistance to certain antibiotics. Most gram-negative bacteria are more inherently resistant to antibiotics than gram-positive because of the presence of the OM [32]. Yet, the OM alone is inadequate to cause this intrinsic resistance but also concomitantly requires the expression of the efflux pumps [32]. Expression of the efflux pumps, which extrude the drugs out of the bacterial

cell, is one of the major determinants of antibiotic resistance. The genes encoding for the drug efflux pumps are chromosomally encoded but can also be acquired. They can also be constitutively expressed or inducible and such pumps can be substrate-specific or have a broad spectrum of substrates [33, 34].

1.5. Conclusion

To overcome the resistance problem, new approaches are needed. There must be a constant pipeline of novel antibiotics to overcome the antibiotic resistance problem. One approach to tackle antibiotic resistance is to alter the strength the action of prevailing antibiotics so that the enzymes of bacteria that cause resistance cannot interfere with its action. An alternative approach to the antibiotic resistance problem is to modify the action of the efflux pumps. Efflux pumps extrude the drugs out of the bacterial cell. By inhibiting the action of these pumps, resistance problem can be attainable. Another approach is to use of combination therapy. Combination of antibiotics might have synergistic effect in the activity of antibiotic. Lastly, one of the ways to overcome the resistance is to develop antibiotics that inhibit novel targets. One such target is the *de novo* purine biosynthesis pathway. In 1980s, it was found that the human and bacterial purine biosynthesis pathways are different. The pathway in bacteria requires 11 steps to synthesize inosine monophosphate (IMP) and involves 2 additional enzymes whereas in humans both of them are absent [35]. The differences between human and bacterial *de novo* purine biosynthesis pathway makes this pathway an ideal target for the antibiotic drug discovery and development.

CHAPTER 2: Introduction

2.1. Overview of the *de novo* purine biosynthesis pathway

The purine biosynthesis pathway is an important pathway for the survival of bacteria. Purine nucleotides are essential in many aspects of cellular metabolism. These include the structure of DNA and RNA, serving as enzyme cofactors, functioning in cellular signaling, acting as phosphate group donors, and generating cellular energy. Maintenance of the proper balance of pools of these nucleotides inside the cell is critical to normal function.

The *de novo* biosynthetic pathway for purine nucleotides is highly conserved among organisms, but its regulation and the organization of the genes encoding the enzymes vary. This pathway was found by Buchanan et al. in 1950s [36]. Purine biosynthesis requires ten enzymatic transformations to generate inosine monophosphate (IMP) from phosphoribosyl pyrophosphate (PRPP) [37]. IMP is converted to guanosine 5′-monophosphate (GMP) or adenosine 5′-monophosphate (AMP) by subsequent enzymes (Figure 6).

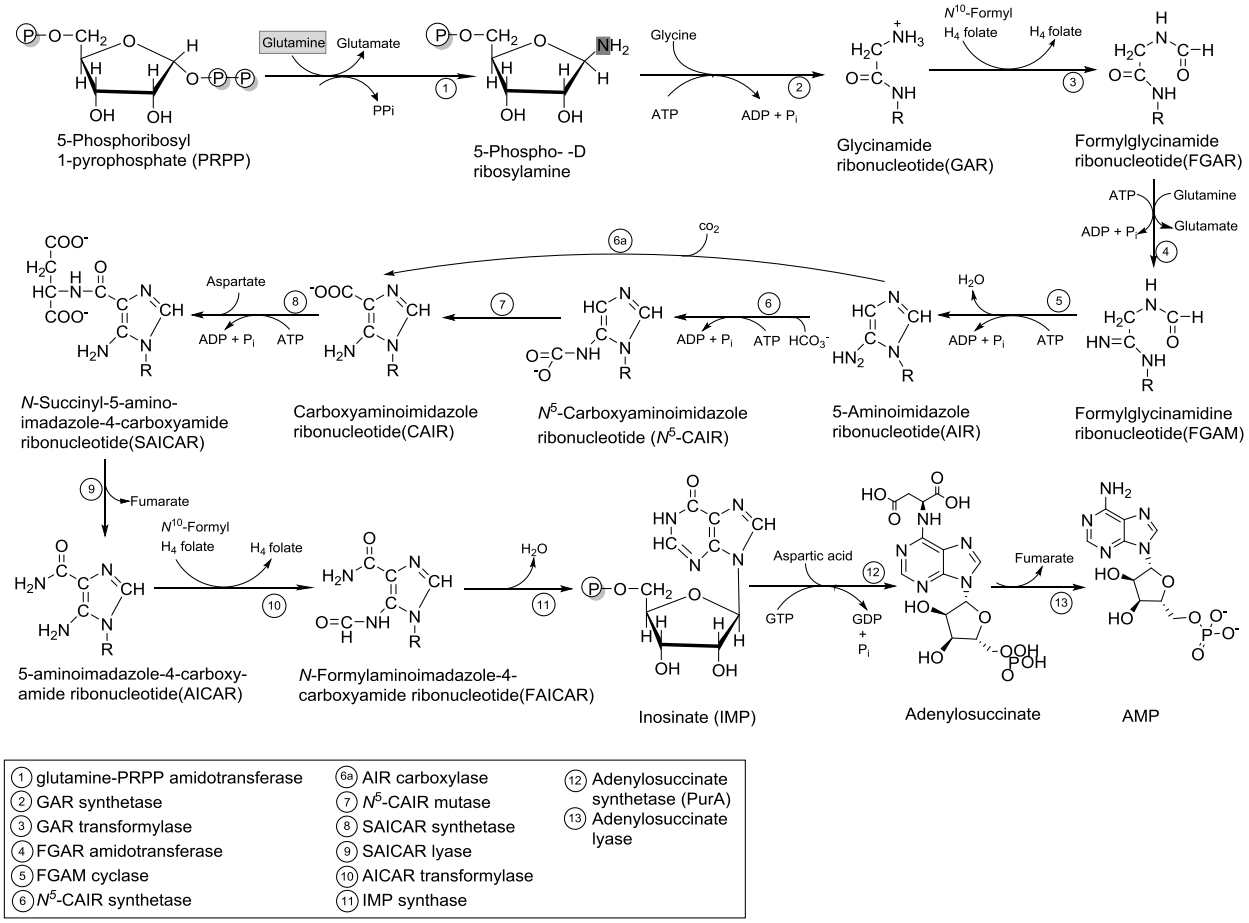


Figure 6. Bacterial Purine Biosynthesis Pathway

The research in 1990s showed that there were differences in purine biosynthesis pathway between higher eukaryotes (e.g., humans) and that of bacteria and yeast. It was found that "enzyme PurF, PurD, PurL, PurM, PurC, and PurB are common to all pathways, while PurN or PurT, PurK/PurE-I or PurE-II, PurH or PurP, and PurJ or PurO catalyze the same steps in different organisms[37]. These variations are not only found at protein level but are also found in gene organization. For example, in *E. coli* all of the enzymes except those for steps 9 and 10 are separate gene products, while in higher organisms the enzymes for steps 6 and 7 are fused, as are

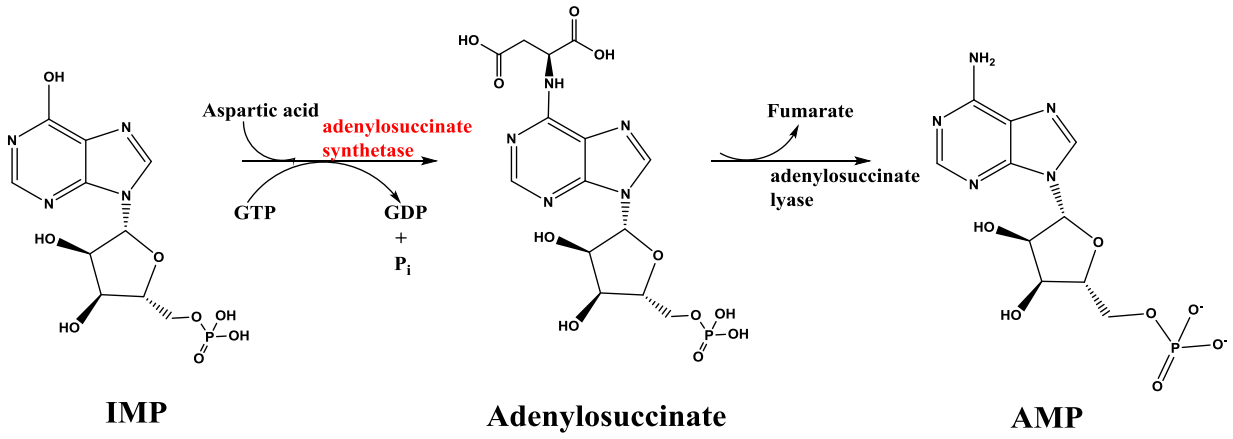
the enzymes for steps 2, 3 and 5" [37]. In addition, it was previously stated that the fusion of these several enzymes might be essential for substrate channeling [38].

The second pathway for purine synthesis is the salvage pathway which recycles purine bases generated during metabolic degradation of nucleotides. However, this pathway produces only 1% or less of the total nucleotides needed for DNA synthesis [39]. Therefore, de novo purine biosynthesis is the major process for generating purine bases needed for replication of organisms.

Because of the important role of purine nucleotides in RNA and DNA synthesis, the *de novo* purine biosynthetic pathway is considered an important target for antiviral and antimicrobial drug development.

2.2. An introduction to Adenylosuccinate Synthetase or PurA

The Adenylosuccinate Synthetase (PurA) is a component of purine nucleotide cycle and governs the first committed step in the purine biosynthesis of adenosine monophosphate (AMP).



Because of its crucial role in synthesis of AMP, PurA is present in mostly all life forms, from archaebacterial [40] and bacteria [41] to mammals [42, 43]. The enzyme from *Escherichia coli* is by far the best-characterized enzyme [44, 45].

2.2.1. Enzyme Structure and Conformational changes

"The crystal structure of PurA from *E. coli* reveals that the dominant structural element of each monomer of the homodimer is a central β-sheet of 10 strands (Figure 7). The first nine strands of the sheet are mutually parallel with right-handed crossover connections between the strands. The 10^{th} strand is antiparallel with respect to the first nine strands. In addition, the enzyme has two antiparallel β-sheets, composed of two strands and three strands each, 11 α-helices and two short 3/10-helices" [45].

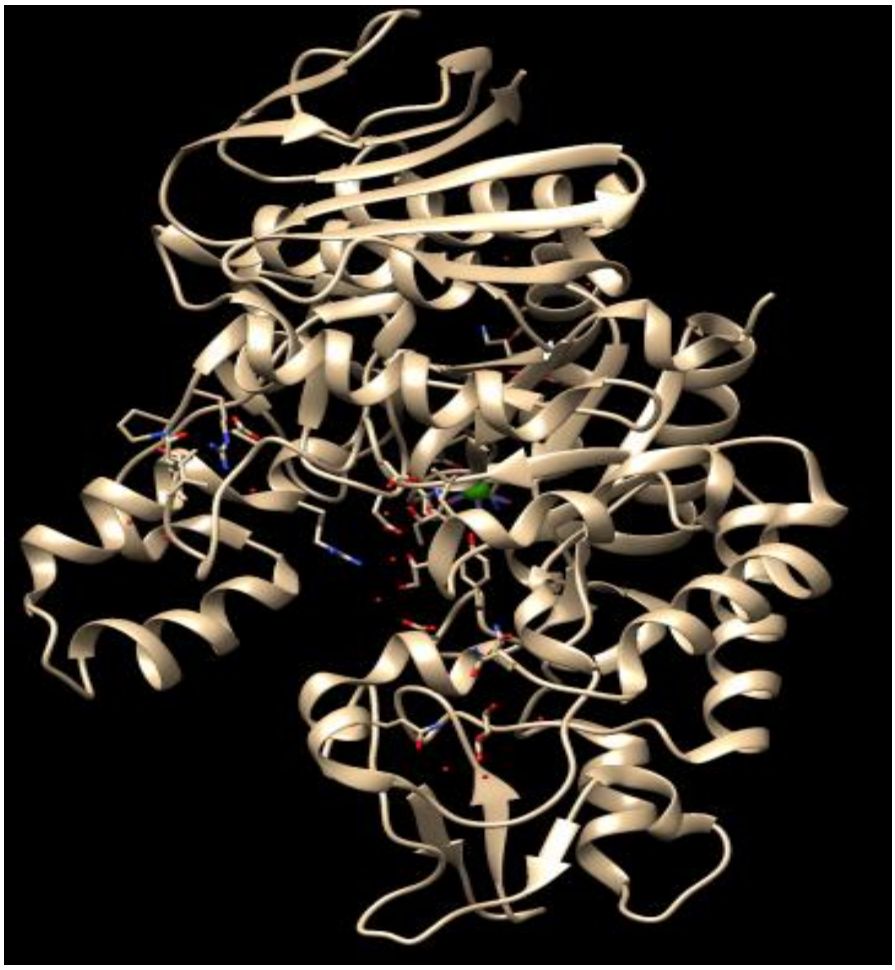


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Figure 7. Structural conformation of *E Coli* PurA

"The individual strands of the β-sheet are connected by segments of the polypeptide chain that range in size from short loops to subdomains made of several elements of secondary structure. The ligand binding causes conformational changes that are involve in the movement of these connecting elements toward the active site" [45-47]. "Residues 38-53 (40s loop), residues 120-130 (120s loop) and residues 299-303 (300s loop) are part of the most important conformational changes in the enzyme. The 40s loop undergoes a 9 Å displacement towards the active site upon

binding. The 120s loop and the 300s loop do not show well-defined conformations in the absence of ligands and gets compact into ordered conformation upon binding of the active site."

2.2.2 Ligand binding sites of PurA

The active site of the enzyme is the place where the substrate binds and at which catalysis occurs. Figure 8 shows the interactions between substrates and active site residues in PurA [47]. A middle Mg^2 interacts with GDP, with ligands near the aspartic acid site, and with ions that occupy the γ-phosphate site. "The enzyme structure surrounds the Mg^{2+} coordinated complex with its three substrates. Although IMP is not coordinated to Mg^{2+} initially, the putative 6-phosphoryl-IMP intermediate becomes an inner sphere ligand of the metal cation. The chemical changes mediated by the synthetase depict an "isomerization" of this Mg^{2+}-coordinated complex" [47]. Identification of active site residues is important in drug discovery process. The structure of the enzyme can be analyzed to identify active site and design drugs that can fit into them.

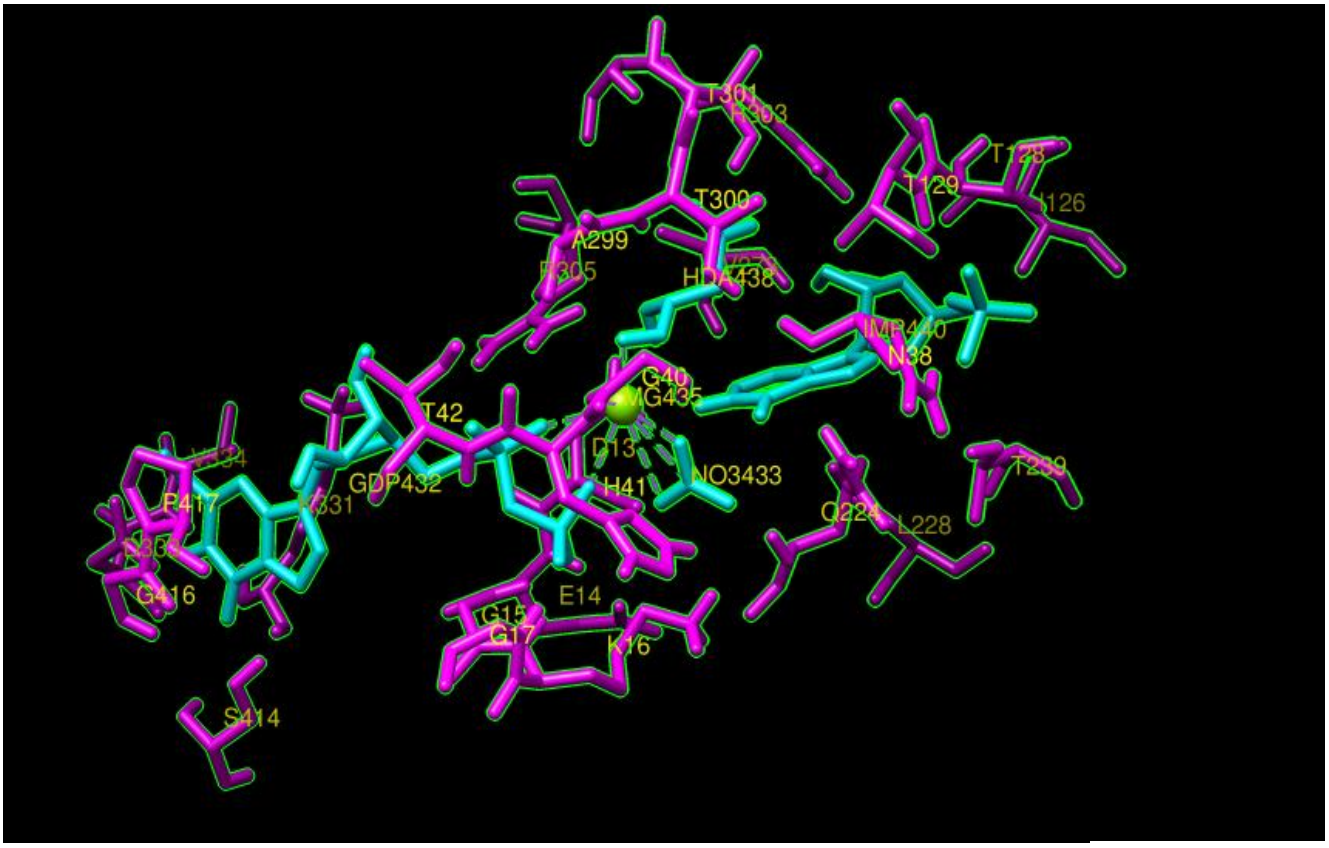

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Figure 8. Ligand binding site of PurA. The ligands are shown in cyan and active site residues are shown in pink.

2.2.3 Inhibitors of PurA

Many different compounds have been tested as *E. coli* PurA inhibitors (Figure 9). Following are some of the inhibitors of *E. coli* PurA.

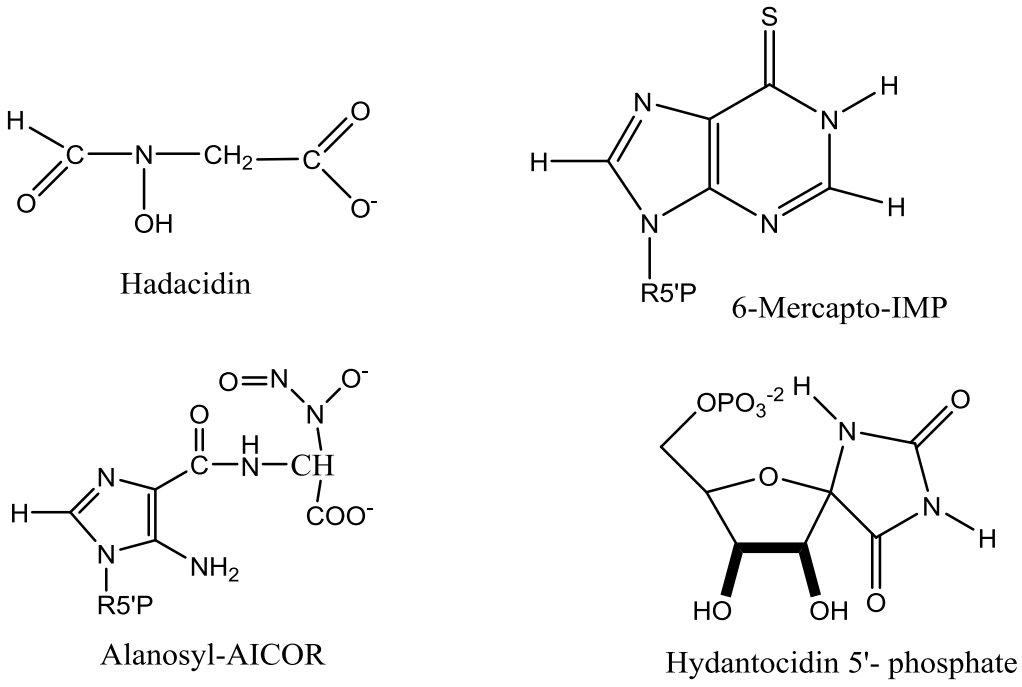

Figure 9. Known inhibitors of *E coli* adenylosuccinate synthetase

Apart from above, AMP, GDP and GMP are known to cause feedback and product inhibition of PurA. In addition, AMPS is a good inhibitor, with K_i ranging from 5-30 µM for the enzyme from different bacterial sources. Hadacidin, a natural antibiotic, is also one of the inhibitor of PurA [47-49].

2.3. PurA as antibacterial target

The difference, described above, provide a significant rationale for targeting PurA as antibacterial target. This contention has also been supported by various research studies at genetic level on purine auxotrophs. These studies have shown that the PurA enzyme is important for bacterial survival inside the host. Colony forming units for bacterial strains mutated for purA

gene were observed to be significantly reduced as compared to the wild type strain when grown in serum without supplementing it with adenine [50]. The study done by G. Ivanovics et al on *B. anthracis* purA auxotrophs showed that they were unable to cause virulence in murine model of infection. None of the adenine-dependent strains was capable of killing mice even when exogenous dose of adenine. Reversion to prototrophy caused full virulence and killed the mice[51]. Other researchers have also shown the importance of purine biosynthesis pathway in bacterial virulence [52-54]. These studies validate the hypothesis that adenylosuccinate synthetase (PurA) is a good target for the development of novel antibiotics.

In this thesis, various strategies to identify novel lead inhibitors against *B. anthracis* will be presented. First, the purA gene was cloned and enzyme was expressed and purified. MESG assay was then employed to determine the substrate kinetics for PurA. Substrate kinetics was determined to know the substrate concentration required for significant catalysis of the reaction and to identify the competitive inhibitors that compete with substrate to bind to the free enzyme. Subsequently, malachite green assay was optimized for high throughput screening of 75,000 compounds to identify inhibitors against PurA. The hits were validated and assay interference from the hits from high throughput screening was corrected using the artifact assay. To confirm the hits from artifact assay, an independent binding assay was performed to determine the binding and equilibrium dissociation constant of these compounds.

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CHAPTER 3: Cloning, Expression and Purification of PurA

3.1 Introduction

Molecular cloning refers to the process of making multiple molecules. The copied material, which has the same genetic makeup as the original, is referred to as a clone. It is used in a wide array of biological experiments and practical applications ranging from genetic fingerprinting to large-scale protein production.

Molecular cloning of any DNA fragment generally involves following steps:-

1. Fragmentation - Breaking apart a strand of DNA and amplification usually done by PCR.
2. Ligation- Pieces of DNA are glued together in a desired sequence.
3. Transfection- The newly formed pieces of DNA are inserted into competent cells.
4. Screening/Selection - The cells that were successfully transfected with the new DNAare selected.

3.2 Materials and Methods

3.2.1. Molecular Cloning of PurA

Bacillus anthracis ΔANR strain was chosen for getting the genomic DNA. PurA gene has 1290 base pairs or 430 amino acids. The gene was amplified from this genomic DNA using the polymerase chain reaction technique. A 100 µL PCR mix consisted of 2 µL each of 10 µM forward primer 5'-GCGGAGGTGCTTTAATACATATGTCTTCAGTAG-3' and reverse primer 5'-CTCAAAAATATCAACTCGAGTTAAGCTTCGTATACGTTA-3', 1 µL of genomic DNA, 10 µL of 10X PCR buffer, 1 µL of 50 mM dNTP mix, 2 µL of Taq polymerase, and 82 µL of

molecular biology grade water. The reactions conditions were as follows: initial denaturation (94 °C for 120 s); 35 cycles of denaturation (92 °C for 30 s), annealing (56 °C for 30 s), and elongation (68 °C for 90 s). The PCR products were analyzed by electrophoresis in a 0.8% agarose gel made in TBE buffer (Tris, borate, EDTA) stained with ethidium bromide and analyzed by UV light. The PCR product was cleaned with a QIAquick PCR Purification kit.

The purified PCR product and the purified vector (using the QIAprep Spin Miniprep Kit) were then double digested separately. First, they were digested for 2 hrs by NdeI (Fast Digest, Fermentas) at 37 °C and then with by Xho1 (Fast Digest, Fermentas) for 1 hr at 37 °C. Purification of digested PCR product and digested vector was done using QIA Quick PCR Purification kit and samples were run on a gel to analyze digestion. Ligation was followed after digestion. The protocol provided by the Roche rapid ligation kit was used. The vector/insert molar ratio was kept 1:1 for ligation.T4 DNA Ligase was used to ligate insert and vector and reaction was carried out for 30 minutes.

E. coli competent cells (BL21 strain) were used for transformation by heat shock method at 42 °C for 30s, and transformed cells were incubated overnight at 37 °C on LB agar plates with ampicillin(100 µg/mL). Individual colonies were selected and the presence of the insert in the vector was analyzed by PCR and double digestion by the restriction enzymes *Nde*I and *Xho*I. Results were visualized on an electrophoresis gel (1 % agarose). The purified DNA of the positive colony (having the insert in its vector) was sent for sequencing.

3.2.2 Protein Expression and Purification

The clone for purA gene stored in BL 21 (DE3) cells were grown overnight in around 10 mL LB medium with 100 µg/µL ampicillin shaking at 220 rpm at 37 °C. 500 mL flasks of TB with 100 µg/µL ampicillin were inoculated by the overnight culture. Cultures were grown at 37 °C at 220 rpm until the OD_{600} reached 0.5 and then IPTG was added (final concentration 0.5 mM) for induction and cultures were continue to grow for an additional 4 hours. Induced cells were harvested by centrifugation at 8000 rpm for 20 min (Beckman, JA-10 rotor). The cell pellets were then suspended for 1 hr into the lysis buffer (approximately 12 times the weight of cells; 50 mM Tris, pH 8.0, 500 mM NaCl, 10 mM Imidazole, 1 % Triton X-100, 0.025 mg/mL DNase I, 1 mg/mL lysozyme, protease inhibitors- 250 µL for every 5 g of cells). After lysis, the pellet is subjected to sonication (Sonic Dismembrator Model 500, Fisher Scientific) at 65% amplitude for 6 min with a pulse of 3 sec on and 6 sec off to disrupt cell membranes and release cellular contents. The crude extract was then centrifuged for 20 min at 18,000 rpm to separate the soluble and the insoluble fractions. The supernatant was collected and filtered using a 0.22 µm membrane filter and was ready to load into 5-mL Histrap HP affinity column. The Histrap column was first equilibrated with binding buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole). The column was then subjected to ten column volumes of binding buffer to wash unbound impurities and elution was done with buffer B (50 mM Tris pH 8.0, 500 mM NaCl, 500 mM imidazole) using a step gradient of 5%, 10%, 20%, 30%, 50%, 75% and 100% buffer B. Samples were collected in 3 mL fractions and were analyzed by SDS-PAGE for PurA. Required elution peaks were pooled together and dialyzed overnight in dialysis buffer (50mM Tris, 100mM NaCl, 1mM DTT, pH8.0) at 4 °C in order to get rid of imidazole in the sample fractions.

Finally, protein was stored as a glycerol stock (with 12 % glycerol) at – 80 °C. Protein concentration was determined using Nanodrop and Bradford assay.

3.3 Results and Discussion

3.3.1. Cloning and transformation - After transformation, individual colonies were selected and the presence of the insert in the vector was analyzed by PCR and double digestion by the restriction enzymes NdeI and XhoI. Figure 10 show the digestion products of PCR and the plasmid vector using *Nde*I and *Xho*1. Lane 5 shows the presence of insert and the DNA sample from that colony was sent for DNA sequencing to confirm the successful transformation.

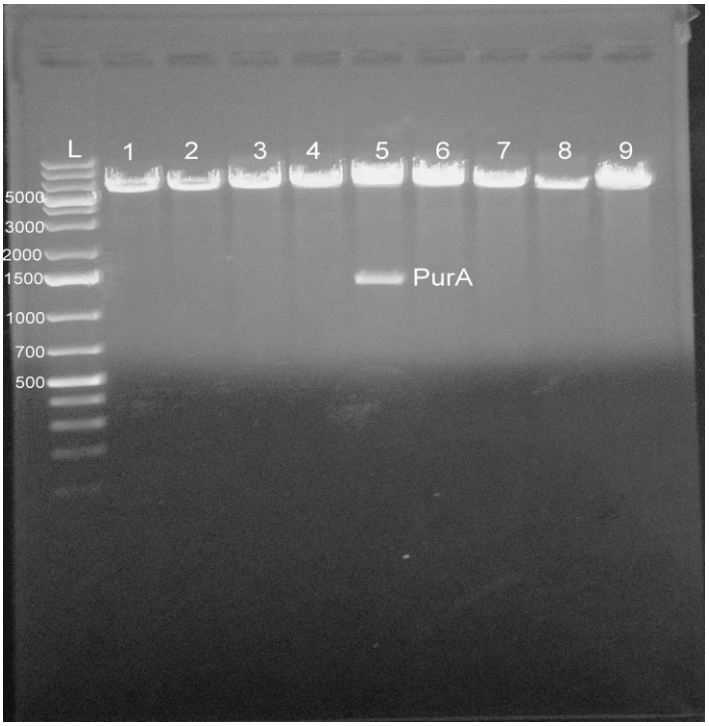


Figure 10. Agarose gel analysis of the restriction digestion products. Lane L- GeneRuler 1 kb Plus DNA Ladder, Lane 1-9 – digested products of PCR from nine different colonies. Lane 5 shows the presence of PurA that is around 1300 base pairs.

3.3.2. Purification of PurA – Figure 11. shows the chromatogram of purification of PurA using HisTrap affinity chromatography. Two peaks were observed which were eluted at 30 % and 40 % of elution buffer B respectively.

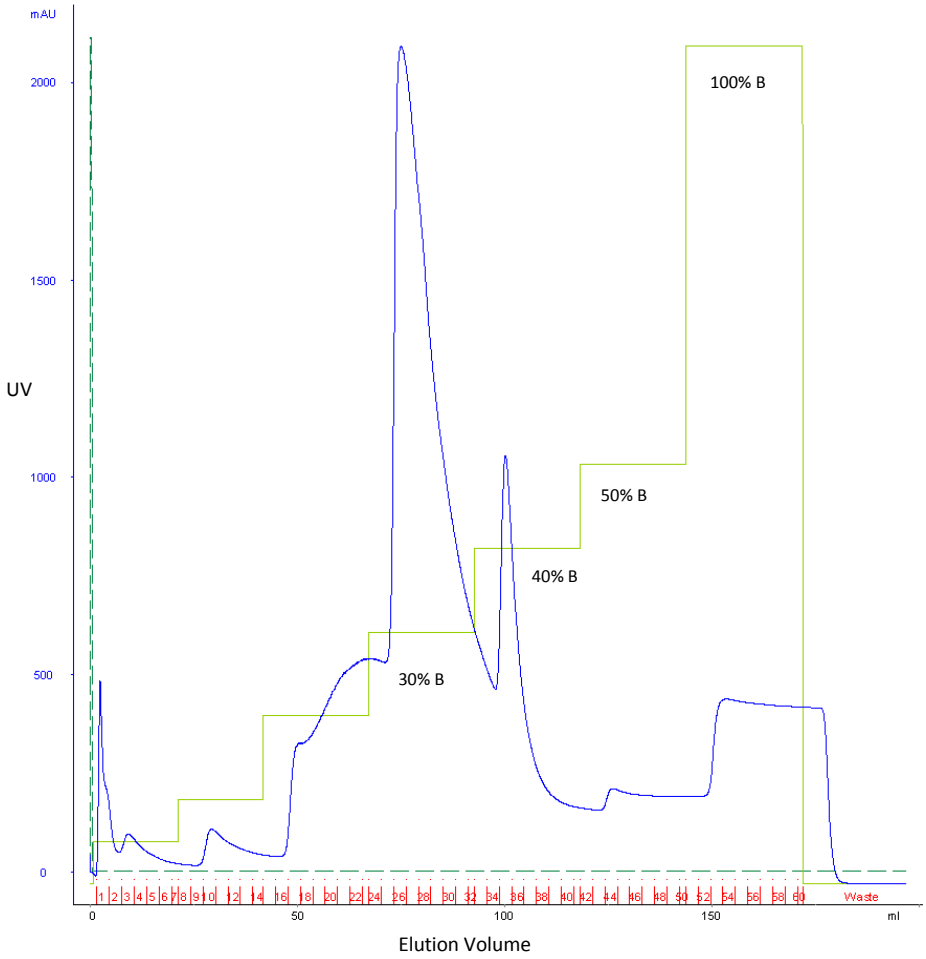


Figure 11. Chromatogram for purification of PurA.

SDS-PAGE analysis was performed to confirm the presence of PurA in the two peaks observed from affinity chromatography (Figure 12). Fractions from the peak contained a single band just below the 49 kDa marker, which is approximately equal to the predicted molecular weight of 47

kDa from the sequence of PurA. In addition, the protein was confirmed and tested for activity using the malachite green assay.

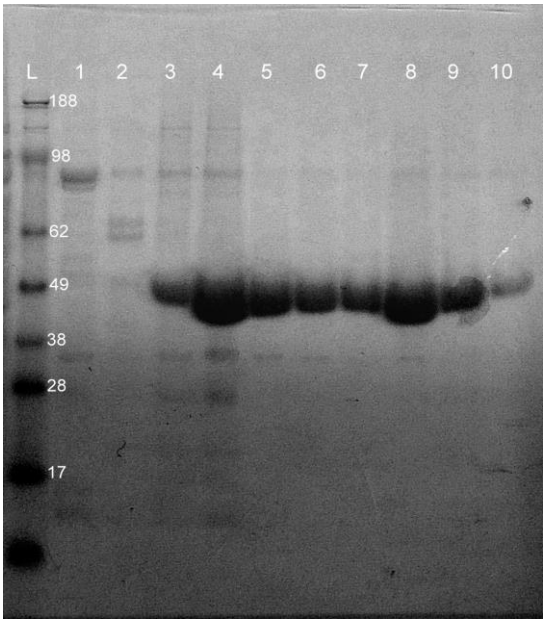


Figure 12. SDS-PAGE analysis of fractions from affinity chromatography. Lane L ladder, lane 1 flow through, lane 2 unbound wash, lane 3-7 fractions from peak 1, lane 8-9 fractions from peak 2, lane 10- fraction from 50% elution buffer B.

CHAPTER 4: Substrate kinetics for the *B anthracis* Adenylosuccinate Synthetase

4.1 Introduction

Reactions with multiple substrates follow complex rate equations that describe how the substrates bind to the enzyme and in what sequence. The analysis of these reactions is simple if the concentration of one substrate is kept constant and other substrates are varied. Under these conditions, the enzyme behaves just like a single-substrate enzyme and a plot of rate v Vs substrate concentration [S] gives apparent K_M (Michaelis Menten constant)and V_{max} (maximum velocity) constants for the other substrates.

One of the important parameters affecting the rate of an enzyme-catalyzed reaction is the substrate concentration, [S]. Therefore, it is necessary to determine the substrate concentration to know about the mechanism of binding of the substrates to the enzyme. There are three types of enzyme inhibitors – competitive, uncompetitive, and non-competitive (or mixed type) [38]. Competitive inhibitors compete with the substrate for binding to the free enzyme and therefore, higher concentration of substrate will not favor the formation of the enzyme-inhibitor EI complex. As a result, assay with high substrate concentration will be biased against detecting competitive inhibitors and if the substrate concentration is below the K_m, even weaker competitive inhibitors will be detected easily. Uncompetitive inhibitors bind only to the enzyme-substrate ES complex. Therefore, higher substrate concentration will result in higher ES complex, which will favor the binding of these inhibitors to the ES complex. As a result, assay with higher substrate concentration will be biased for uncompetitive inhibitors to be easily be detected in the assay. The third type of inhibitors is called the non-competitive or mixed-type

inhibitors. This type of inhibitors binds to both the free enzyme as well as the ES complex. Therefore, this type of inhibitors is less sensitive to [S].

In an enzymatic reaction, the initial velocity V_i gradually increases with increase in substrate concentration. Finally a point is reached, beyond which the increase in V_i will not depend on the [S]. A graph with [S] on the X-axis and corresponding V_i on Y-axis indicates that as the [S] increases, there is a corresponding increase in the V_i (Figure 13). However, above a certain [S], the velocity of the reaction remains constant without any further increase. The maximum velocity of the reaction under saturation of substrate is known as the V_{max}, and concentration of substrate where the velocity of reaction is half the maximum velocity is known as K_m (Michaelis constant).

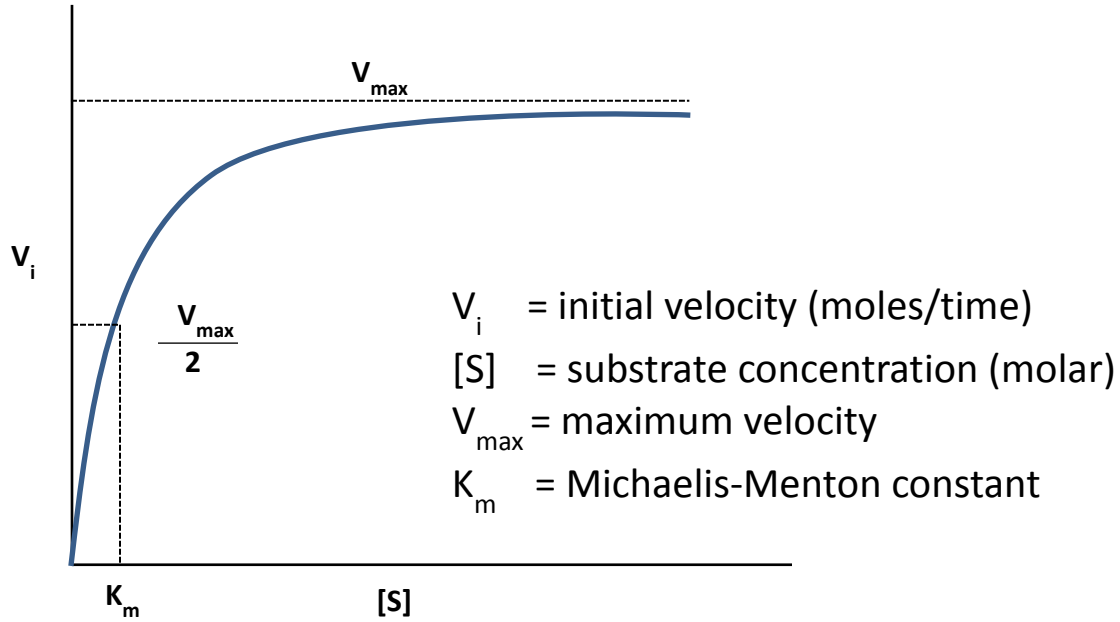


Figure 13. Kinetics for enzyme to determine K_m

4.2 MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside) assay

The MESG assay was used to determine enzyme kinetic parameters. This assay measures the rate at which inorganic phosphate is released in an enzymatic reaction.

PurA catalyzes the following reaction:

IMP + Aspartate + GTP $\longrightarrow$ Adenylosuccinate + GDP + phosphate (Pi)

In the above reaction, there are three substrates – inosine monophosphate (IMP), guanosine triphosphate (GTP) and Aspartic acid. The K_m values for each of the three substrates – IMP, GTP and Aspartic acid was determined using MESG assay. MESG assay is a continuous assay and can be used to measure the rate of phosphate release from enzyme catalyzed reactions [55]. PurA catalyzes the reaction to form the product adenylosuccinate, guanosine diphosphate (GDP) accompanied by the release of inorganic phosphate (Pi). The substrate MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside), is converted into ribose 1- phosphate and 2-amino-6-mercapto-7-methylpurine in the presence of P_i by the enzyme PNP (Purine Nucleoside Phosphorylase). The product 2-amino-6-mercapto-7-methylpurine has absorbance maximum at 360 nm (Figure 14). This absorbance is proportional to amount of inorganic phosphate formed which is proportional to rate of the enzyme reaction.

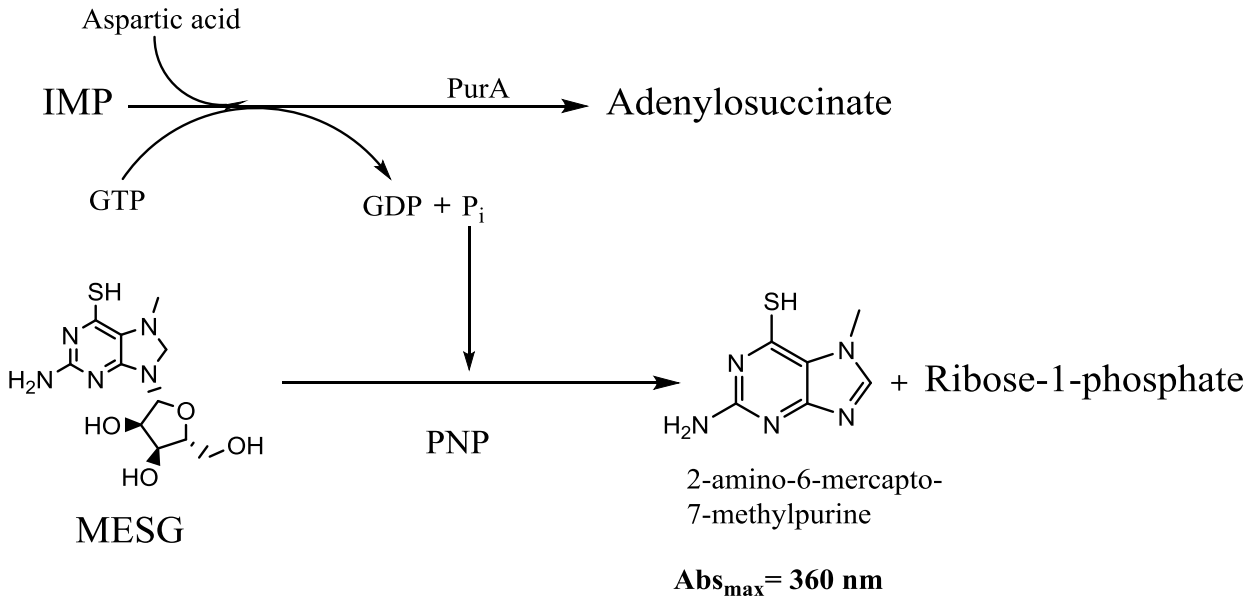


Figure 14. MESG assay for adenylosuccinate synthetase

4.3 Materials and Methods

4.3.1. Materials

The EnzChek® Phosphate Assay Kit for MESG assay was purchased from Life Technologies. Tris- HCl, NaCl, $MgCl_2$, IMP and aspartic acid were purchased from Sigma.

4.3.2. Method for MESG assay

The K_m value for each of the substrates – IMP, GTP and Aspartic acid was determined by double substrate kinetics. The assay was performed in a 96-well plate format with an assay volume of 200 µL. Each assay well contained 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.2

µM PurA enzyme, 0.5 U PNP, 300 µM MESG and varying concentration of substrate- IMP, GTP and Aspartic acid. The above reaction was incubated for 5 minutes and initiated by the varying concentration of substrate. The measurement of first set of enzyme reaction rate was made by varying GTP and aspartic acid concentration and fixing the IMP concentration. The second set of measurements was made by varying IMP and GTP concentration and fixing the aspartic acid concentration. For the third set of measurements, concentration of IMP and aspartic acid was varied (5-100 µM) and GTP concentration was kept constant. The data were fit to the Michaelis-Menten equation:-

$v = V_{max}[S]/(K_m + [S])$

where v is the reaction rate (µmole/min/mg), V_{max} is the maximum reaction rate (µmole/min/mg), [S] is the substrate concentration (µM), and K_m is the Michaelis-Menten constant (µM). The data was fit into the above equation using the enzyme kinetics module in SigmaPlot and Lineweaver-Burk curve was plotted.

4.4 Results and Discussion

The kinetic data were represented as Lineweaver-Burk plots of the reaction rate vs the substrate concentration. For each plot, one substrate concentration was kept constant and other two substrates concentration was varied (Figure 15-17).

GTP vs Aspartic acid Kinetics:-

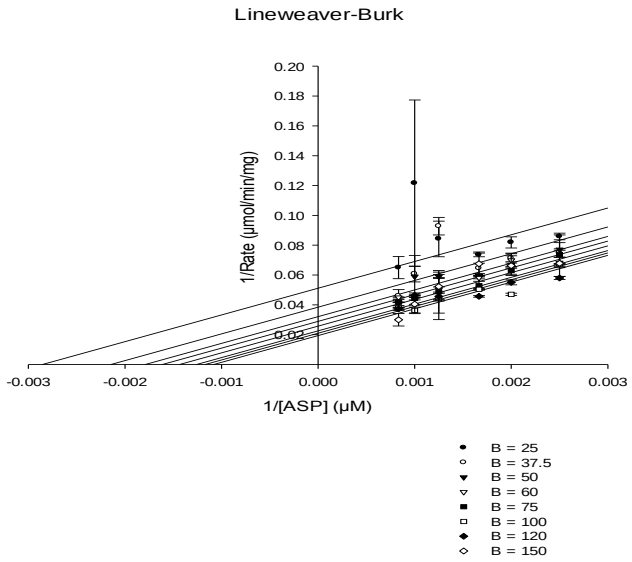


Figure 15. Double reciprocal plot of the reaction rate against the concentration of varied concentration of GTP and Aspartic acid and IMP was kept constant.

GTP vs IMP kinetics:-

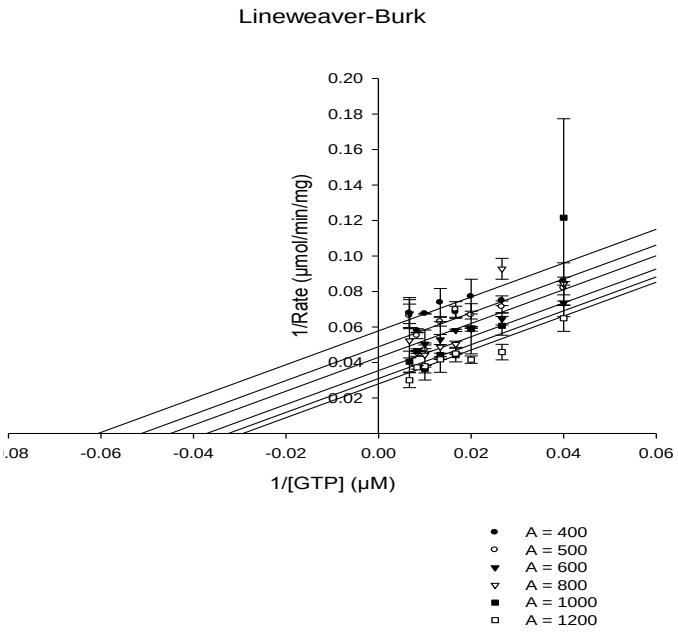


Figure 16. Double reciprocal plot of the reaction rate against the concentration for the varied concentration of GTP and IMP and Aspartic acid was kept constant.

IMP Vs Aspartic acid kinetics:-

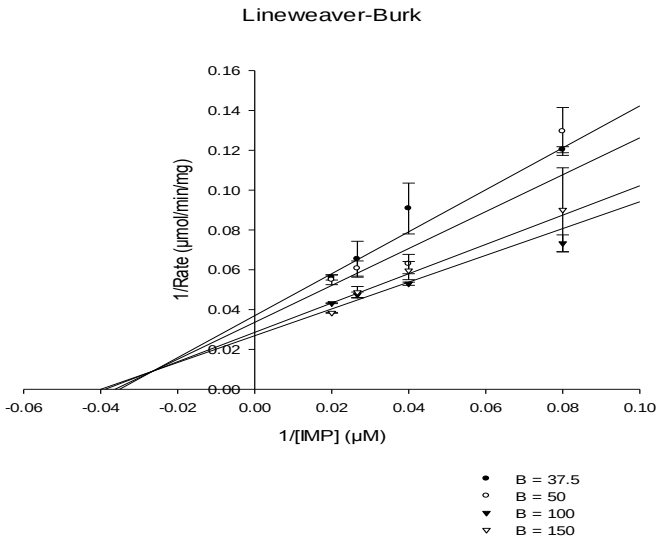


Figure 17. Double reciprocal plot of the reaction rate against the concentration for the varied concentration of IMP and Aspartic acid and GTP was kept constant.

TABLE I. THIS TABLE SUMMARIES THE K_m FOR ALL THE THREE SUBSTRATES

Substrate	K_m (µM)
IMP	32.0
GTP	76.5
Aspartate	936

It was necessary to determine the kinetics for all the three substrates, as we wanted to identify the competitive inhibitors. Competitive inhibitors compete with the substrate for binding to the free enzyme and therefore, higher concentration of substrate will not favor the formation of the

enzyme-inhibitor EI complex. As a result, assay with high substrate concentration will be biased against detecting competitive inhibitors and if the substrate concentration is below the K_m, even weaker competitive inhibitors will be detected easily. Moreover, we wanted to determine the substrate kinetics for significant catalysis of reaction. The K_m values from *B. anthracis* PurA were found to be comparable to that of from *B.subtilis* [56].

CHAPTER 5: High Throughput screening for novel inhibitors of *B. anthracis* Adenylosuccinate synthetase

5.1. Introduction to High Throughput Screening (HTS)

High throughput screening (HTS) is a popular approach to validate the target as it allows the assaying or screening of a large number of potential biological modulators against a chosen set of defined targets. It capitalizes automation to quickly assay the biological or biochemical activity of a large number of drug-like compounds. HTS brings together expertise in liquid handling and robotic automation, multi-platform plate readers and this combinatorial approach means that data can be produced over a relatively short space of time. The advantage of HTS allows rapid, inexpensive, and effective scaling of small scale bench assays to large scale assay formats. High- Throughput screening can be used for many different purposes: the discovery of lead chemical structures for novel drug targets, including ion channels, GPCRs, kinases, and transporters. The results of these experiments provide data which could be used as a starting point for drug design and development and for understanding the interaction of a particular biochemical process in biology.

5.2 Development of assay for HTS

In order to screen for potential inhibitors for *B anthracis* PurA, it was necessary to use an assay that could be adapted to an automated system. Ideally, an assay for the high throughput screening would be as follows:

(1) Be easy to perform and be sensitive.

(2) Less complex and easy to automate.

(3) Work over a time scale of several minutes to facilitate screening of thousands of compound libraries.

(4) Reduce background signal from presence of the compounds tested.

MESG assay was not an ideal assay to be used for HTS of compound libraries. MESG assay is a continuous assay and the readout for assay is at 360 nm. Compound libraries consist of compounds that can interfere at shorter wavelengths at around 340 nm- 400 nm that can lead to false positive and false negative results. Therefore, an assay that has readout at high wavelength is preferable.

Colorimetric assays have become a common assay to check enzyme activity and fulfill the above criteria for use in robust HTS. Malachite green assay was conducted to screen compound libraries for finding potential inhibitors against PurA.

5.3. Malachite Green assay for PurA

Malachite Green Phosphate Assay provides a simple colorimetric method for the determination of inorganic, soluble phosphate concentrations. Malachite green assay is an end point assay for detection & quantification of Pi [57]. It is based on reaction of phosphomolybdate with malachite green to form a green colored complex with an absorbance maximum at around 630 nm (Figure 18). The absorbance at 630 nm is proportional to the amount of Pi which is proportional to the enzyme activity at fixed substrate concentration in the assay.

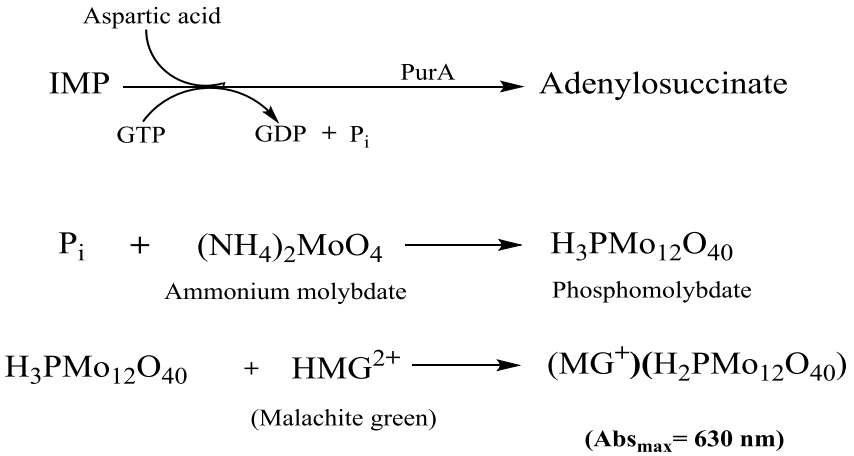


Figure 18. Malachite Green assay for HTS against PurA

Various advantages of Malachite green assay make it a suitable assay for HTS. Malachite green assay has high sensitivity and can detect as low as 1 µM of free Pi. Moreover, Malachite green assay is not expensive. It also requires no enzyme coupling which and makes assay less complex. Compounds can interfere with assay signal at short wavelength around 340- 400 nm which can lead to false positive and false negative results. Malachite green assay uses detection at around 630 nm which reduces the chances of compounds interference. Lastly it can readily be automated on HTS system and reagents can last long for a year.

5.4. Materials and Methods

IMP, GTP and Aspartic acid were purchased from Sigma. Malachite green powder and ammonium molybdate were purchased from Sigma Inc. All other reagents were of highest commercial grade.

5.4.1. Preparation of the Malachite Green dye solution

The Malachite green dye solution was prepared as described [58]. 120 mL of 36N sulfuric acid was added to 600 mL then cooled down to room temperature to make malachite green stock solution. This stock solution is stable for 1 year at least at room temperature [58]. The working malachite green dye solution was made by mixing 5 mL of malachite green solution with 1.25 mL of the 7.5% (w/v) ammonium molybdate solution and 0.1 mL of the 11% (v/v) tween-20 solution. For 384-well plate based HTS assay format, 70 µL of the assay volume in each well was mixed with 17.5 µL of the above working malachite green dye solution (ratio 4:1).

5.4.2. Standard curve preparation and sensitivity check for Malachite green assay

A standard curve was plotted by measuring the absorbance of the solution at different concentration of Pi and quenching the reaction by adding malachite green reagent. For standard curve, 17.5 µL of malachite green dye mix was added to 70 µL of the standard Pi solution at different Pi concentration in the buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl. The sulfuric acid in the malachite green dye causes pH of around 5.0 in the well which stops the reaction immediately after adding the dye. In order to determine the range of free Pi concentration, absorbance was measured at 623 nm of the different concentration of Pi. The assay was performed in a 384-well plate. After 8 minutes of color development, the plates were read in a SpectraMax plate reader. The standard curve was plotted by plotting absorbance on Y axis and Pi concentration on X-axis. The standard curve indicated a linear curve from 1 µM to 25 µM (Figure 19).

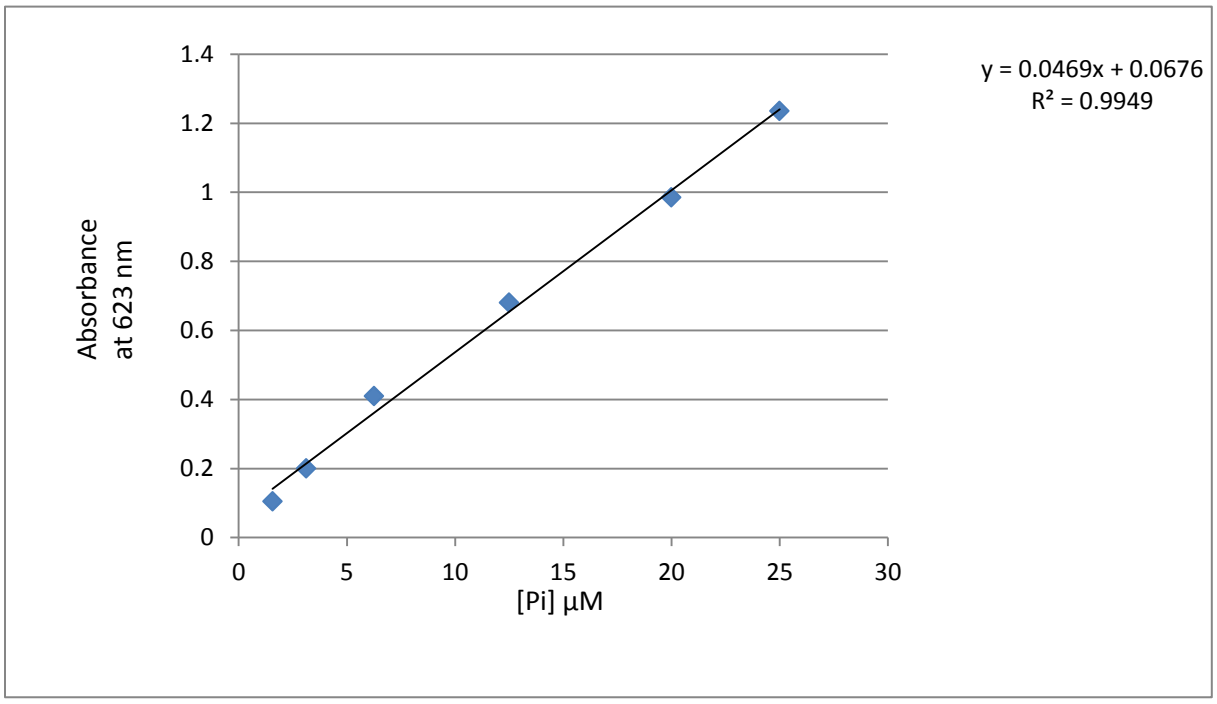


Figure 19. The standard curve of P_i concentration Vs absorbance at 623 nm in the malachite green assay.

5.4.3. Optimization of malachite green assay for various additives

5.4.3.1. Effect of Buffer

Buffers have profound effects on the tertiary and secondary structure of protein which might effects the catalytic activity of the enzyme. For this reason, different types of buffer were tested for the enzyme reaction. The buffer tested were – Tris + NaCl, Tris+HCl, HEPES+NaCl and HEPES+HCl at pH 8.0. The enzyme reaction was carried out at different concentration of IMP. Absorbance at 630 nm was plotted against varied concentration of IMP for different types of buffer used.

5.4.3.2. Effect of Mg^{+2} concentrations

PurA enzyme requires Mg^{+2} to catalyze the reaction to form adenylosuccinate. Effect of different concentration of Mg^{+2} was observed on the assay because excess of magnesium can form phosphate salts that have low water solubility and thereby interfering with assay. Enzymatic reaction was carried out at different concentration of IMP. Absorbance at 630 nm was plotted against varied concentration of IMP for different concentration of Mg^{+2}.

5.4.3.3. Assay tolerance to additives

The primary HTS and the assay thereafter needed presence of several additives. A good HTS should show good tolerance to these additives and it should not interfere with the readout of the assay. The additives that were used in the HTS assay included – DMSO, 0.1 mg/mL BSA, 0.1 % Triton X-100. Compounds to be tested are dissolved in DMSO, so it was necessary to check the effect of DMSO on the assay. The reaction was carried out in different concentration of DMSO to check for its interference with the assay. BSA is added for stability of enzyme in the assay. Detergent such as triton breaks the large aggregates which can be formed by compounds and thereby inhibiting enzyme activity.

5.4.4. Compound Libraries

Two compound libraries were used for the HTS of potential inhibitors against *B anthracis* PurA. The first library was Chembridge containing 50,080 small molecule compounds. Compounds represented in the Chembridge DIVERSet library are carefully selected to provide the broadest pharmacophore space coverage possible within a 50,000 compound set of diverse structures. Several filters have been added to make sure the compounds in the library are competent and are

drug like. These filters include removal of non-drug like compounds and undesirable chemical groups (e.g. Michael acceptors, crown-ethers & analogs, disulfides, epoxides, etc.), molecular weight less than 500, clogP ≤ 5, tPSA ≤ 100, rotatable bonds ≤ 8, hydrogen bond acceptors ≤ 10 and hydrogen bond donors ≤ 5. This library come pre plated into a 384 well format and stored at -30 ^{o}C.

Another library used for HTS was Life Chemicals Library. "This 25,000 compound drug-like chemical library was generated by selecting 18,750 (75%) compounds from the Life Chemicals antibacterial and antiviral activity targeted libraries. The remaining 6,250 compounds were selected from the general Life Chemicals screening collection, which were pre-filtered by the company to be 'Rule of 5' compliant (one exception tolerated). Additionally, a series of custom pre-filters were applied to remove compounds with known reactive functionalities and/or toxicities prior to compound selection" [59]. Molecular weight filters were also employed with a range of 150 to 650 Daltons.

5.4.5. Plate layout for HTS

The HTS assay was performed in a 384-well plate (Figure 20). The first 2 columns (column 1-2) were used as the 100 % enzyme activity control and the last 2 columns (23-24) were used for the 0% enzyme activity control. In the 100 % activity columns, 60 µL of enzyme reaction solution (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM Aspartic acid, 100 µM GTP, 0.1 mg/mL BSA, 1mM TCEP, 0.01% Triton X-100 and 0.35 µM PurA enzyme) was added followed by the addition of 0.2 µL of 100% DMSO. A volume of 10 µL of substrate solution- IMP in final concentration of 35 µM was added in each well to initiate the reaction. In the 0 % activity columns, 60 µL of reaction buffer solution (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM

$MgCl_2$, 1 mM Aspartic acid, 100 µM GTP, 0.1 mg/mL BSA, 1mM TCEP, 0.01% Triton X-100 and no PurA enzyme) was added followed by the addition of 0.2 µL of 100% DMSO and 10 µL of substrate solution as mentioned above. The final concentration of DMSO was 0.29% in the assay. In columns 3-22 were used to test the compound libraries in which 60 µL of enzyme solution was added, 0.2 µL of 10 mM compound was added which resulted in a final concentration of 29 µM of each compound in the assay well and 10 µL of substrate solution was added.

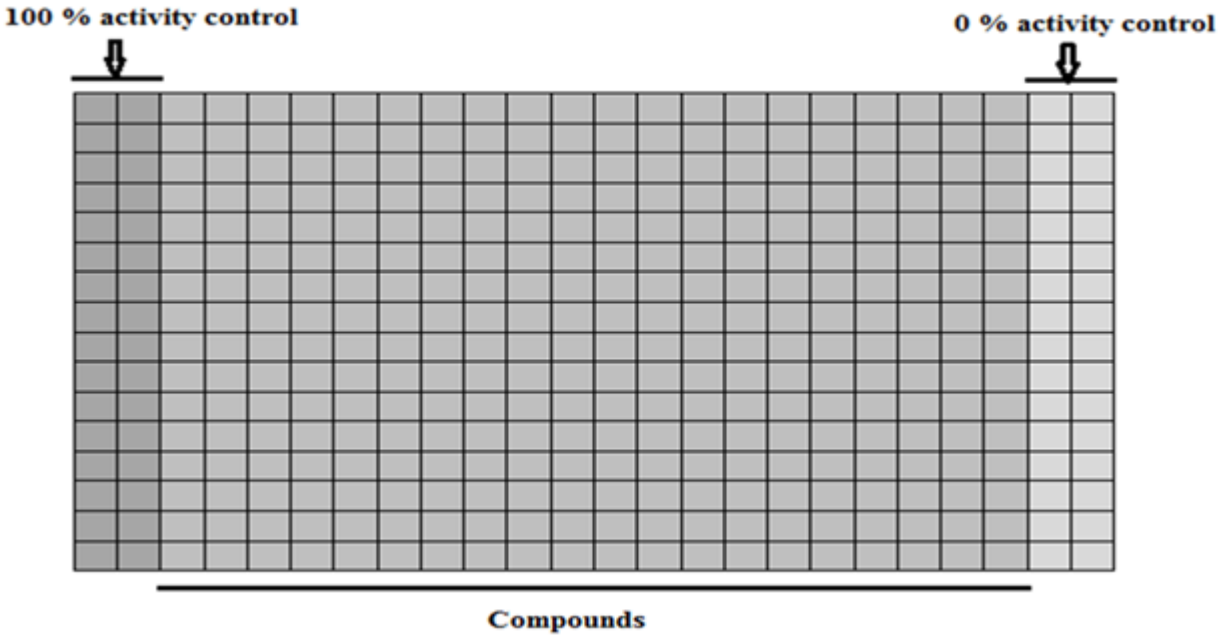


Figure 20. The layout of the 384-well plate for the HTS.

5.4.6. Primary HTS screen of Life Chemicals and Chembridge Compound Library.

Based on assay optimization results, the following protocol was used for HTS against PurA. The enzyme solution containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM

aspartic acid, 100 µM GTP, 0.1 mg/mL BSA, 1mM TCEP, 0.01% Triton X-100 and 0.35 µM PurA enzyme was prepared and 60 µL of this was added into each well from column 1-22 of the assay plate using a Tecan robotic liquid handling machine. In column 22-23, 60 µL of no enzyme reaction buffer solution was added. The compound library was in 384- well format and each compound was 10 mM in concentration. 0.2 µL of this 10 mM compound was added to the assay solution to achieve around ~29 µM concentration in each well. The compounds were tested in duplicates. In 100% and 0% activity wells, no compound was added instead 0.2 µL of DMSO was added. The reaction was then initiated by addition of 10 µL of substrate solution containing 245 µM IMP in water to make final concentration 35µM in each well. The reaction was allowed to proceed for 2 minutes at room temperature, and then reaction is quenched by adding 17.5 µL of malachite green dye solution. The assay solution was mixed by shaking at 1500 rpm for 30 s and then incubated for 5 min for color development. The absorbance for each well was measured at 623 nm (bandwidth: 10 nm) as an end point reading (Figure 21).

Assay Assembly for HTS procedure.

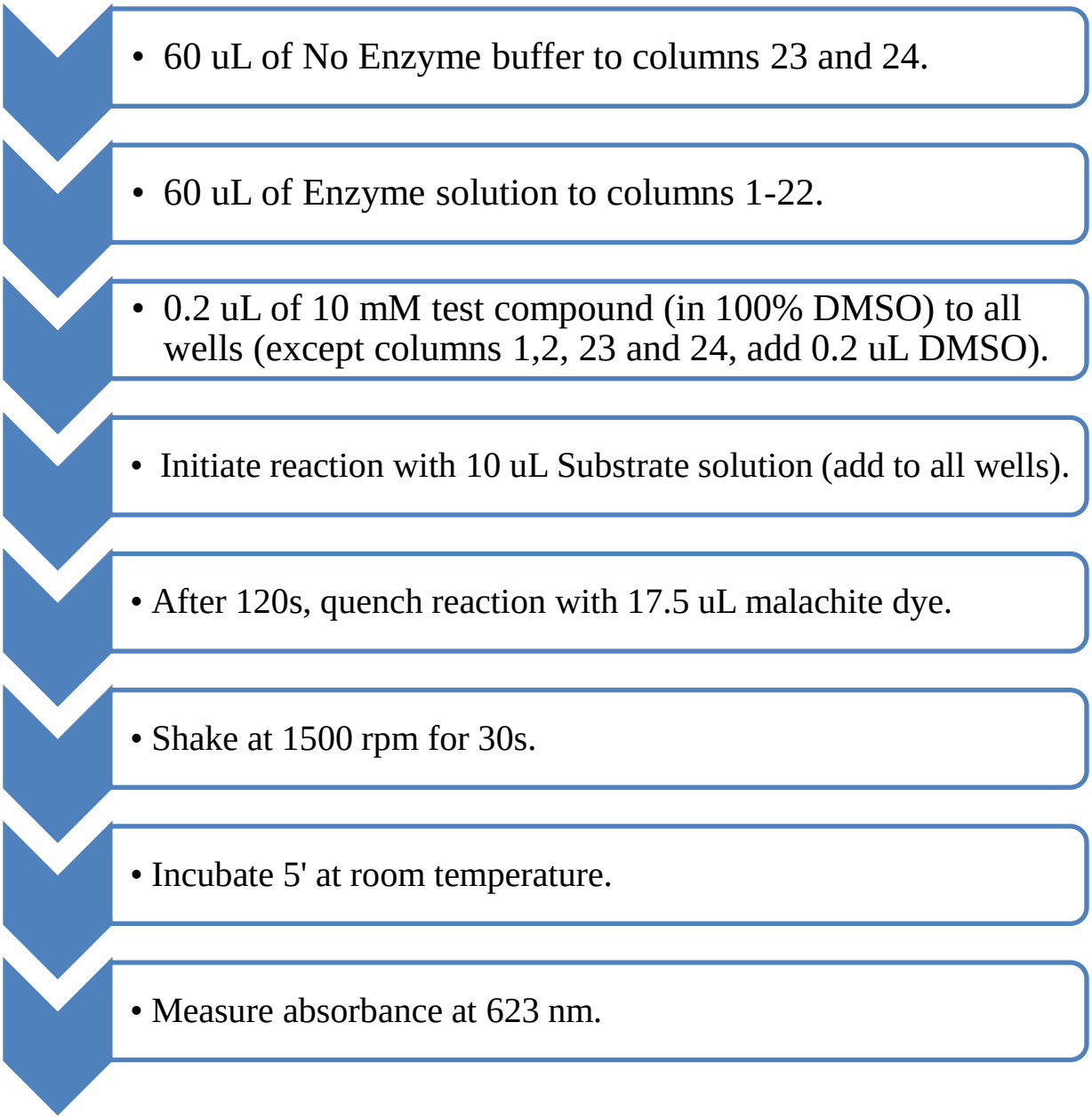


Figure 21. Assay assembly for the HTS

5.4.7. Data Analysis

The data was automatically processed from the plate reader and output as a single file for each library with all the screening data, which is compatible to be used with MS Excel or JChem.

For every plate screened, % inhibition was calculated. % inhibition was calculated as:

$$\% \text{ inhibition} = \frac{\mu_{100} - X}{\mu_{100} - \mu_0} * 100$$

where, X = absorbance of compound

μ_0 = mean of absorbance of 0% activity control wells

μ_{100} = mean of absorbance of 100% activity control wells

Two output files were generated, the first file contained % inhibition and the second file contained the mean and standard deviation of the controls on each plate as well as the Z'- factor values calculated from the controls of the assay. The Z'-factor was calculated for each duplicate screen and is defined as:-

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

where σ_p , σ_n , μ_p & μ_n are standard deviation (σ) & mean (μ) of the 100% and 0% activity controls.

The following are the interpretations for the Z'-factor [60].

Z-factor	**Interpretation**
1.0	Ideal. Z-factors can never exceed 1.

between 0.5 and 1.0	An excellent assay.
between 0 and 0.5	A marginal assay.
less than 0	There is too much overlap between the positive and negative controls for the assay to be useful.

The Z' factor for Malachite green assay was found to be around 0.88 which makes it an excellent assay for the HTS screening.

5.4.8. Hit Selection

The primary screening data were processed using the perl scripts language that was developed in house. The compounds were ranked by the mean % inhibition from the test done in duplicates. For the Life Chemicals Library, compound with mean % inhibition higher than 40 % were classified as hits. For the Chembridge Library, compounds with mean % inhibition of 30% and higher were classified as hits.

5.4.9. Minimal Inhibitory Concentration (MIC) analysis

For minimal inhibitory concentration analysis, *B anthracis* non virulence strain was grown in LB medium until its $O.D_{600}$ was 0.4. After the O.D reached 0.4, compounds were then added to the LB medium in varying concentration from 200 nM to 50 µM of compound. Only DMSO was added to the untreated controls. These were then incubated and grown overnight at 37 ^{0}C. The lowest concentration of compound at which there is no bacterial growth is defined as minimal inhibitory concentration (MIC).

5.4.10 Hit Validation

High Throughput screening against *B anthracis* PurA resulted in total of 65 hits. Out of these 65 hits, we were able to purchase only 27 compounds from Life Chemicals library and 18 compounds from Chembridge library. These 45 compounds were purchased and stored at -30 ^{0}C as 50 mM stock by adding DMSO. The 50 mM stock was then diluted to 10 mM stock in DMSO and it was used as a working stock for future analysis.

The hits from HTS were tested using the malachite green assay at 29 µM of the compound to see where there were true hits and to eliminate the false positive. The experiment was performed using the same conditions used in HTS and mentioned as above. The only difference was that 1µL of compound instead of 0.2 µL of compound, was added to 59 µL of enzyme reaction mix and 10 µL of IMP was used to start the reaction. 17.5 µL of malachite green dye was then used to quench the reaction. Further, the hits from above were analyzed for IC_{50} determination. The compounds were tested at varying concentration (3.125 µM to 100 µM).

5.5. Results and Discussion

5.5.1. Optimization of malachite green assay

Malachite green for High Throughput screening was optimized for different types of buffer conditions, Mg^{+2} concentrations and tolerance of assay for various additives such as DMSO, BSA and Triton X-100.

5.5.1.1. Effect of Buffer

Figure 22 shows the effect of different types of buffer for PurA activity in presence of different concentration of IMP. It was noted that there was no significance difference on absorbance at

630 nm for different types of buffer. Tris + NaCl at pH 8.0 was used as the buffer for HTS screening and further assay as it was used for purification of PurA enzyme and the enzyme was found to be stable in this buffer.

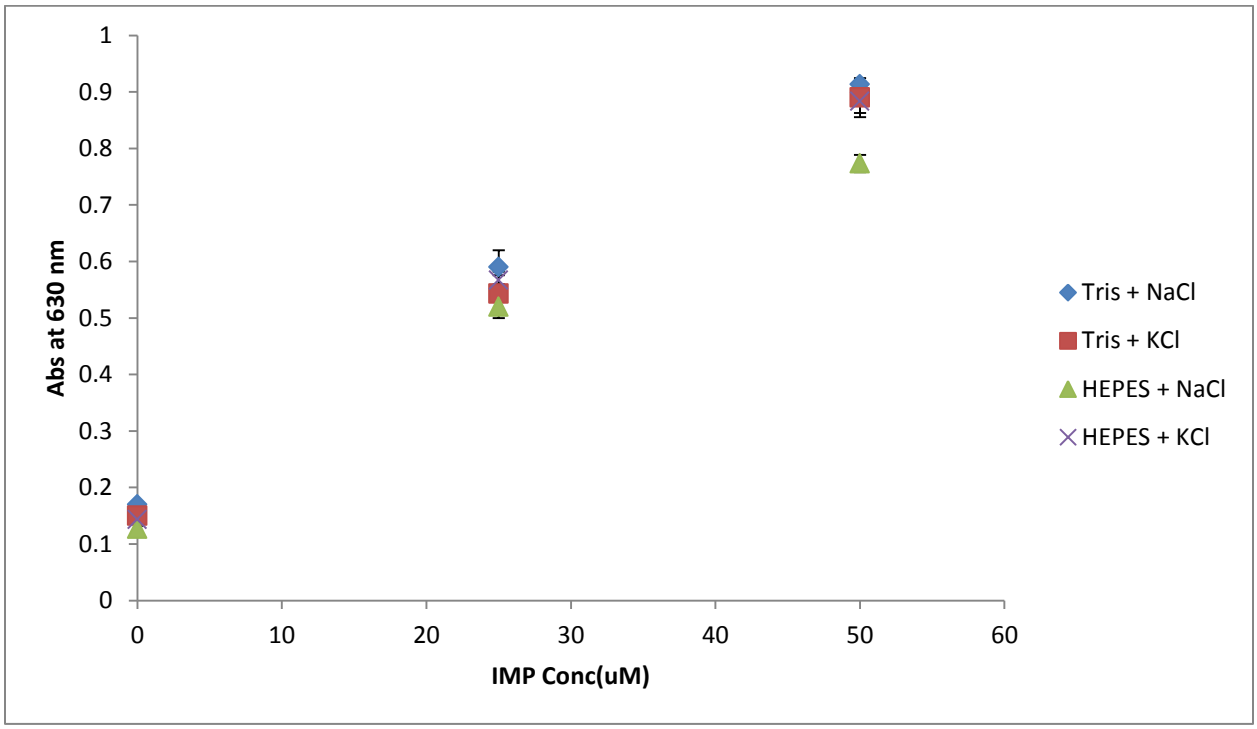


Figure 22. Effect of different buffer conditions on absorbance in malachite green assay. No significant effect was observed of different buffers on absorbance.

5.5.1.2. Effect of Mg^{+2} concentrations

Figure 23 shows the effect of concentration of Mg^{+2} on the absorbance at 630 nm in Malachite green assay. It was observed the absorbance was reduced at high concentration of Mg^{+2}. This might be due to formation of phosphate salts that have low water solubility and thereby interfering with assay. So, 1 mM of $Mg^{+}2$ was chosen as the final concentration for the HTS of compound libraries.

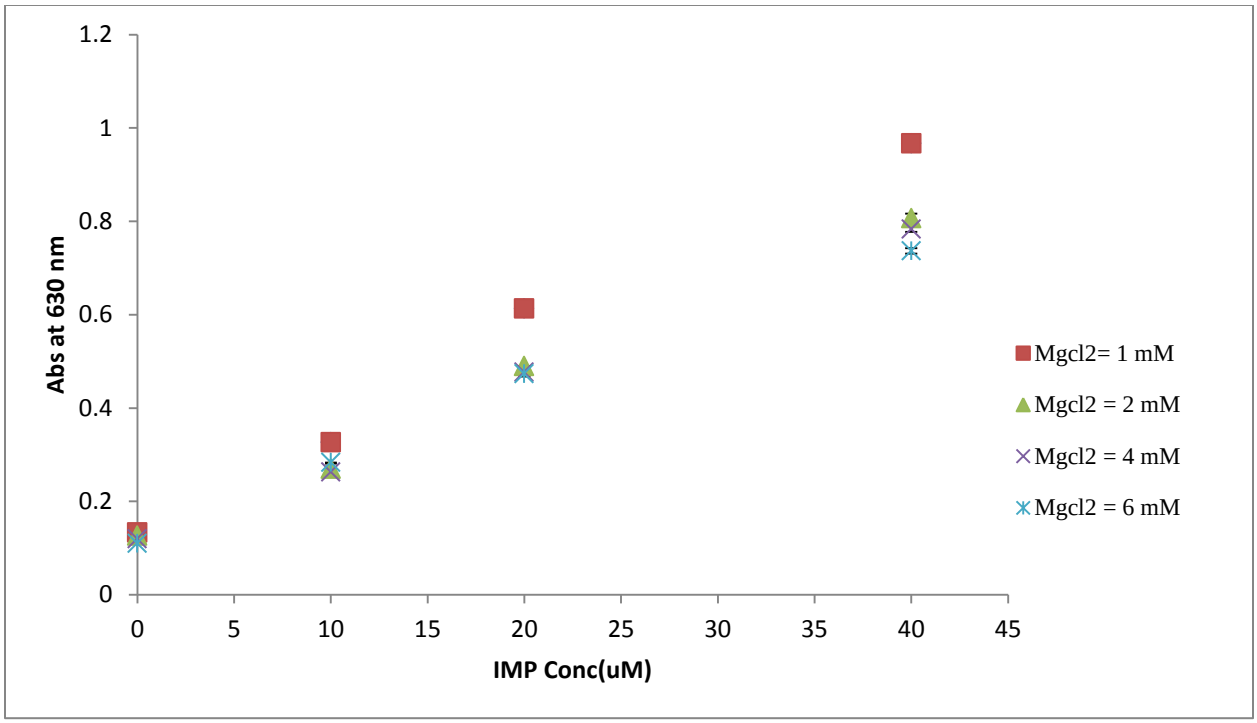


Figure 23. Effect of different Mg^{+2} on malachite green assay. High Mg^{+2} decreased the absorbance.

5.5.1.3. Assay tolerance to additives

As shown in Figure 24, the malachite green assay showed a good tolerance to all the additives tested. DMSO was tested at different concentrations from 0% to 4% and it was observed that there was no significant difference for varying concentration of DMSO. The tested compound had final concentration of 0.02% DMSO.

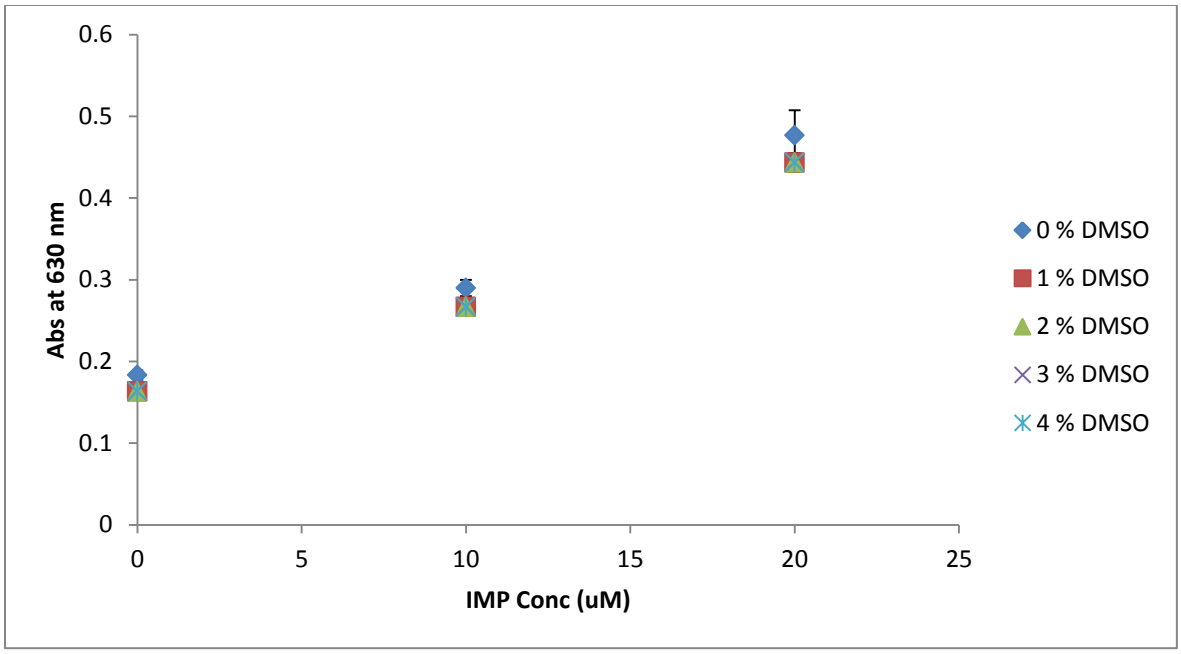


Figure 24. Assay tolerance to different concentration of DMSO.

In Figure 25, effect of additives BSA and Triton at concentration 0.1 mg/mL and 0.01 % respectively on absorbance was observed. Addition of additives had no effect on the absorbance and we decided to go with the concentrations widely used in a HTS setting which is 0.1 mg/mL for BSA and 0.01% for Triton X-100.

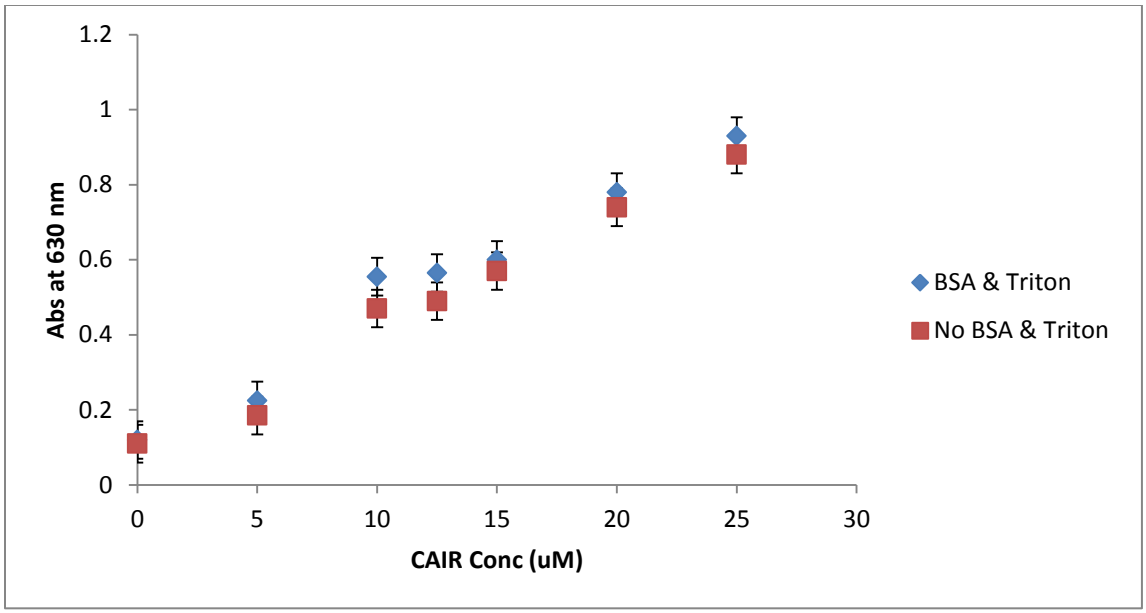
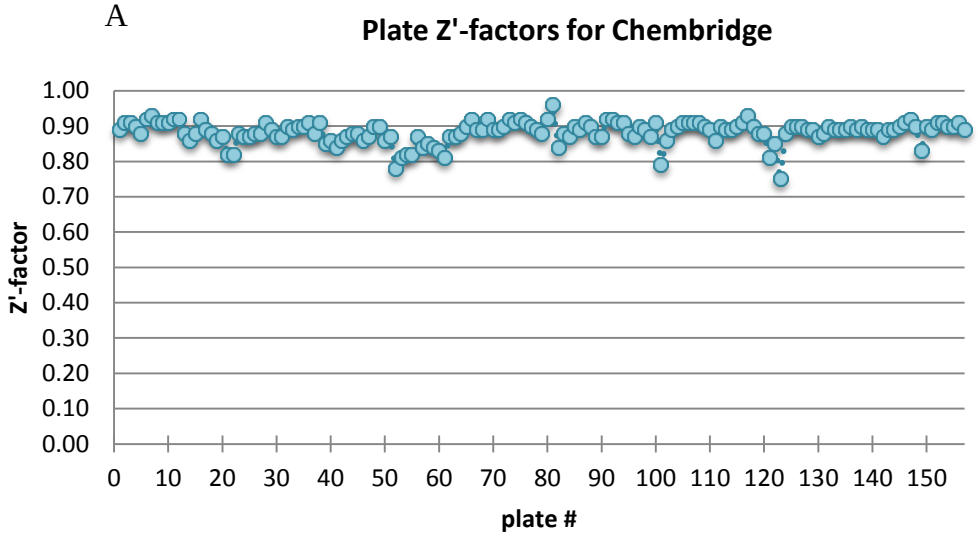


Figure 25. Assay tolerance to BSA and detergent Triton X-100.

5.5.2 Primary HTS screen of Life Chemicals and Chembridge Compound Library.

Two compound libraries namely the Life Chemicals and the Chembridge libraries totaling 75,000 compounds were used to screen *B. anthracis* PurA. The two libraries were screened separately in duplicate in the 384-well plate format. The Z'- factor for every duplicate screen were calculated based on the mean and standard deviation of the controls. The average Z'-factor was 0.88 and 0.89 for Chembridge and Life Chemicals library respectively (Figure 26). The Z'-factor of above 0.8 made it an excellent assay for screening. In addition, for both the libraries, a replication plot for each compound was also generated to check whether HTS was reproducible (Figure 27). For the Life Chemicals library, 40 compounds with mean % inhibition greater than 40% were selected. For the Chembridge, 25 compounds with mean % inhibition greater than 30% were selected. This resulted in a hit rate of 0.09%.

A



B

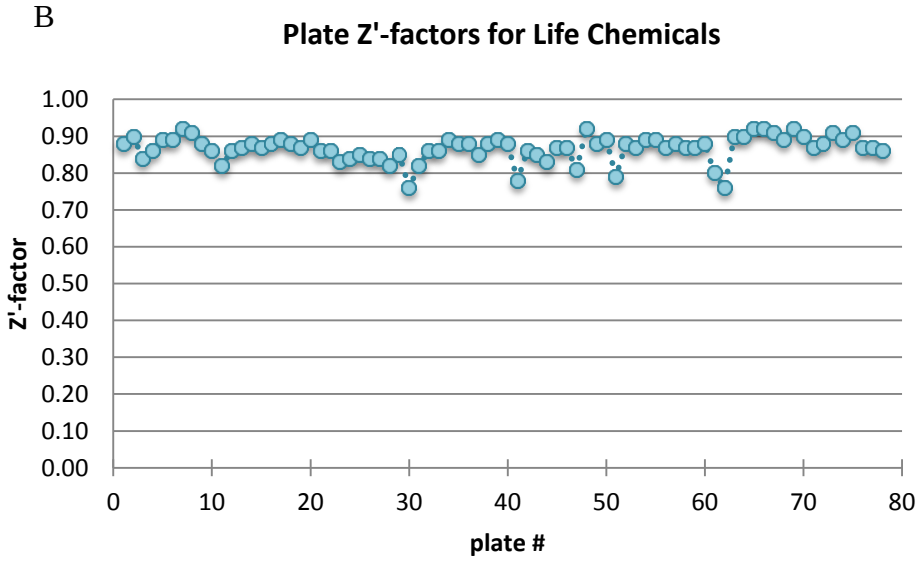
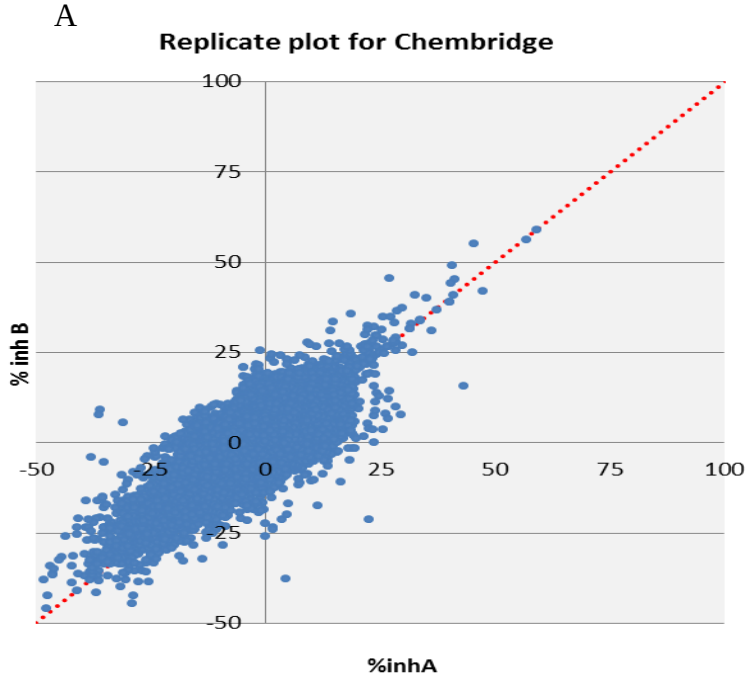


Figure 26. Z'-factor for the compound libraries tested in HTS against PurA.
(A) Z'-factor for Chembridge library. (B) Z'-factor for Life Chemicals library

A



B

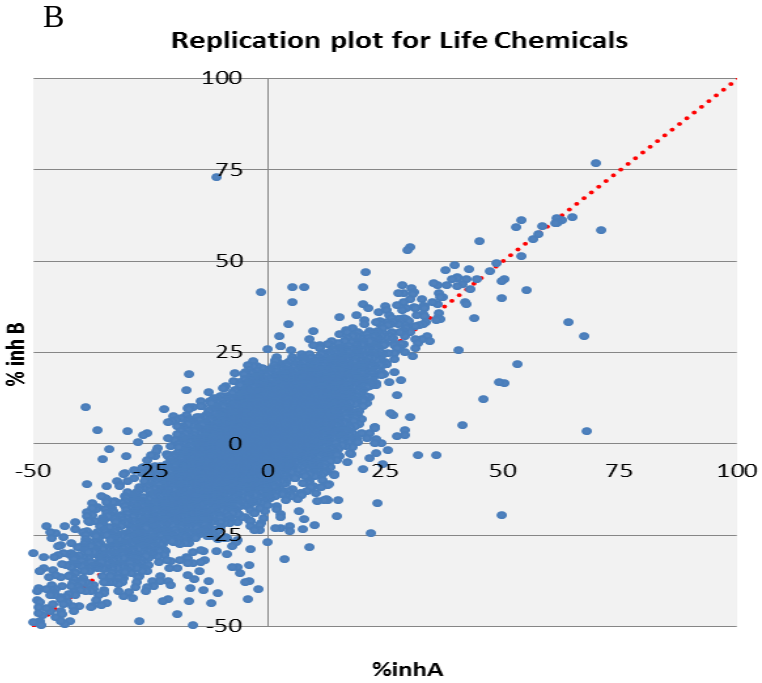


Figure 27. Replication plot for the % inhibtion for each compound in the HTS. (A) The Chembridge Library. (B) The Life Chemicals library.

5.5.4 Minimum Inhibitory Concentration (MIC) Test.

In order to test the anti-bacterial activity of the 45 compounds, MIC was performed on the non-virulence strain of *B. anthracis*. Out of the 45 compounds, 2 compounds from Life Chemicals and 4 compounds from Chembridge showed MIC values less than 12.5 µg/mL. However, we decided to pursue all the 45 compounds for further analysis as we believed the compounds could be tailored for anti-bacterial activity. TABLE II and TABLE III. summaries the MIC against non-virulent strain for the Chembridge and the Life Chemicals library.

TABLE II. MIC AGAINST NON-VIRULENT STRAIN OF *B. ANTHRACIS* FOR THE CHEMBRIDGE LIBRARY

Number	Compound ID	MIC (µg/mL)
1	5150668	>12.5
2	5156968	>12.5
3	5212523	>12.5
4	5312251	12.5
5	5484308	1.56
6	5573985	12.5
7	5672305	>12.5
8	5684237	>12.5
9	5722547	12.5
10	5829408	>12.5
11	7538926	>12.5
12	7705443	>12.5
13	7780701	>12.5
14	7805808	>12.5
15	7925836	>12.5
16	9123184	>12.5
17	9148031	>12.5
18	9149885	>12.5

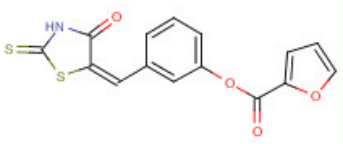
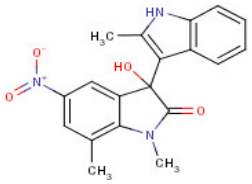
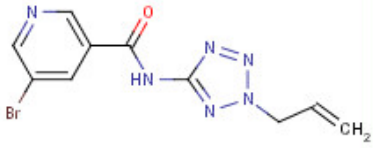
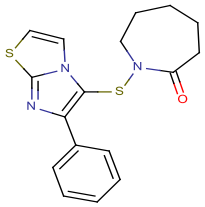
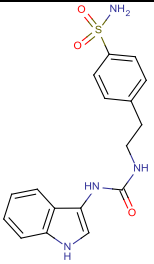
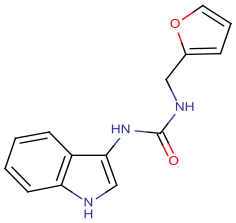
TABLE III. MIC AGAINST NON-VIRULENT STRAIN OF *B. ANTHRACIS* FOR THE LIFE CHEMICALS LIBRARY

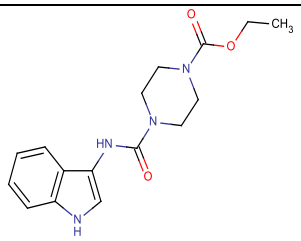
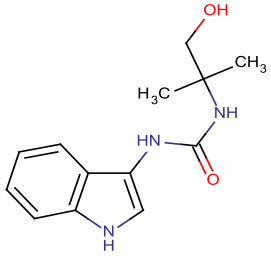
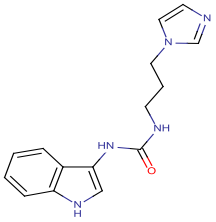
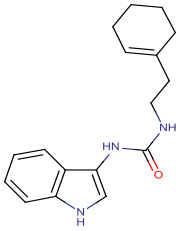
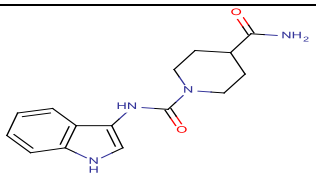
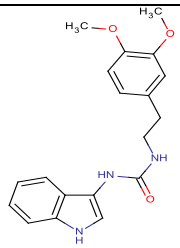
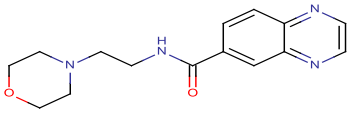
Number	Compound ID	MIC (μg/mL)
1	F1040-0011	2.34
2	F1142-2835	>12.5
3	F1947-0010	6.25
4	F1973-0019	>12.5
5	F2031-0332	>12.5
6	F2732-0096	>12.5
7	F2740-0032	>12.5
8	F2740-0045	>12.5
9	F2740-0079	>12.5
10	F2740-0096	>12.5
11	F2740-0099	>12.5
12	F2740-0113	>12.5
13	F2740-0116	>12.5
14	F2740-0118	>12.5
15	F2740-0133	>12.5
16	F2740-0770	>12.5
17	F3055-0778	>12.5
18	F3055-0799	>12.5
19	F3055-0810	>12.5
20	F3055-0839	>12.5
21	F3211-0056	>12.5
22	F3222-5640	>12.5
23	F3250-0419	>12.5
24	F3305-0011	>12.5
25	F3320-0224	>12.5
26	F3379-0251	>12.5
27	F5123-0875	>12.5

5.5.3. Hit Validation.

From HTS against *B anthracis* PurA, 45 compounds were tested using malachite green assay to determine whether these were true hits. Only 23 compounds, fifteen from Life Chemicals and eight from Chembridge library gave reproducible result (> 40% inhibition for Life Chemicals and >30% inhibition for Chembridge library at 29 µM of compound) with the results from HTS. The compounds that were reproducible were analyzed to determine IC_{50} using the MESG assay. MESG assay is a continuous assay but it was found that this assay was not an ideal to determine the IC_{50}. Similarly the malachite green assay being an end point assay is not an ideal for IC_{50} determination. Instead of pursuing IC_{50} at this stage, we decided to look at the general trend of inhibition in the malachite green assay – higher % inhibition with higher concentration of compound and decided to eliminate the compounds which did not show the above trend. We tested the concentration range from 100 µM to 1.6 µM and found that ten compounds from the Life Chemicals library and three compounds from Chembridge were observed to show the above trend, and were selected for further analysis. In addition, it was interesting to observe that out of 13 compounds, 8 compounds have the same scaffold that had the same indole ring (Figure 28). These 13 compounds were further tested for assay interference using artifact assay. TABLE IV summaries the compounds tested using the malachite green assay and to be tested in the artifact assay.

TABLE IV. SUMMARY OF COMPOUNDS FROM MALACHITE GREEN ASSAY

Compound ID	Structure	% Inhibtion HTS at 29 µM	% Inhibtion Run 2 at 29 µM
5573985		59	38
7780701		37	42
9123184		41	44
F1040-0011		73	56
F2740-0116		61	40
F2740-0113		61	43

F2740-0045		61	49
F2740-0079		62	49
F2740-0099		64	55
F2740-0096		62	42
F2740-0032		58	67
F2740-0118		56	41
F3055-0778		49	50

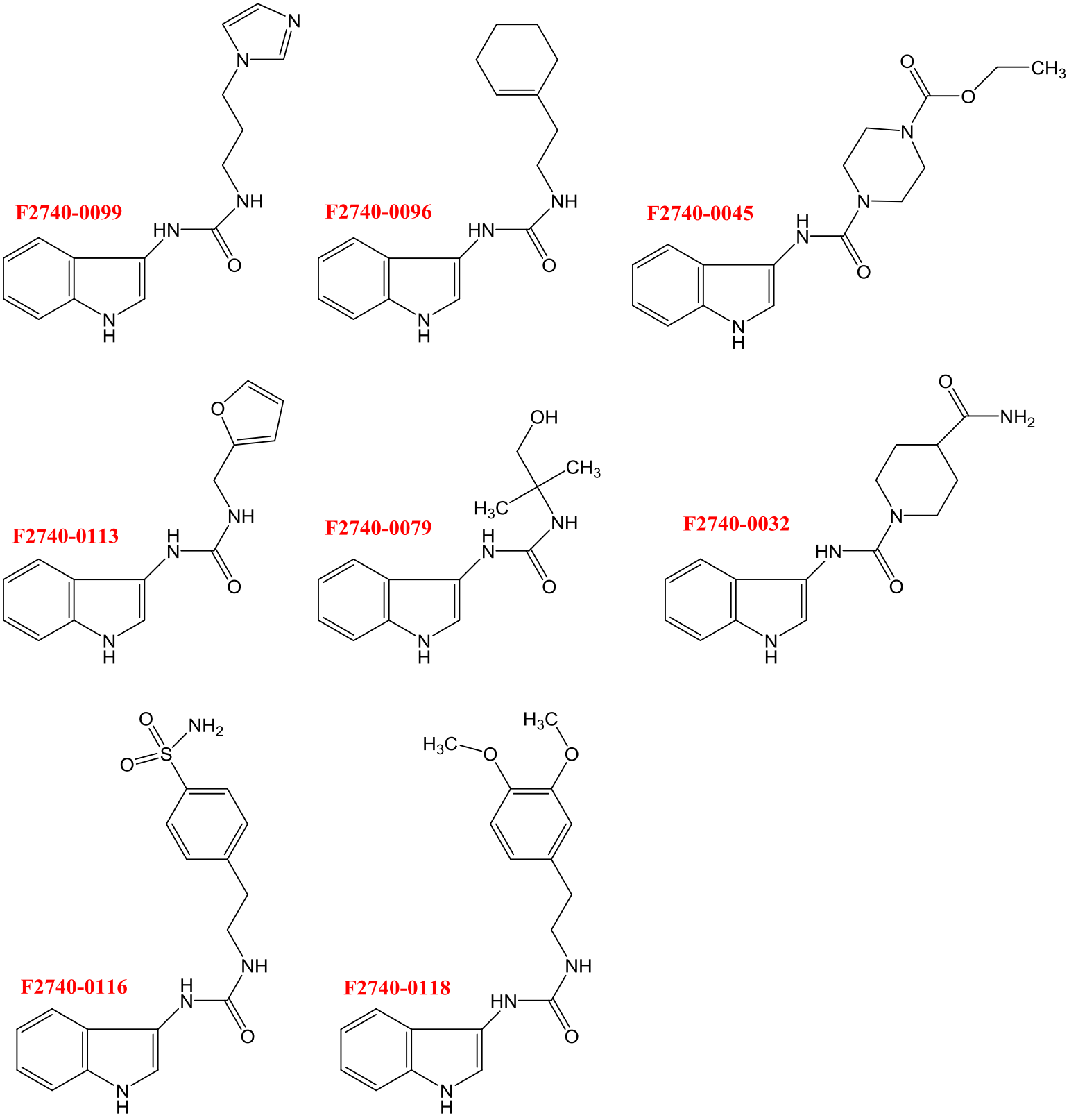


Figure 28. Compounds with same scaffold.

CHAPTER 6: Artifact Assay – Correction for Interference by Compounds in High-Throughput Screening

6.1. Introduction

High Throughput Screening (HTS) is a drug-discovery process widely used in the pharmaceutical industry to screen large collection of chemical compounds libraries. Screens usually involve measuring the inhibitory effect of each test compound on an assay system such as the catalytic activity of an enzyme. The typical readout of the assay is usually a measurement such as absorbance, luminescence, or fluorescence taken for each well of the assay plates. In most of the assays, the components are added at the desired concentration in each well along with the test compound to check for the inhibitory effect on the catalytic activity of the enzyme. A pitfall in these types of assays is the interference of test compounds with the measurement as the test compounds remain in the well during the assay measurement. Below are described some classes of the compound interference that might occur with generally used HTS assay measurements methods.

6.1.1. Interference by absorption of light.

Most of the HTS use absorption of light as the readout of the assay. Compound libraries consist of organic compounds which give UV absorbance in the 200- 300 nm range of the spectrum because of presence of aromatic rings. In addition, aging of test compounds sometimes results in formation of absorbing products which gives absorbance in visible range (360-720 nm). Interference from colored compounds has also been observed. “It is therefore unavoidable that some of the test compounds will have significant absorbance at the wavelength of light recorded in the assay used for compound screening” [61].

6.1.2 Interference by quenching of fluorescence and luminescence

"Assays based on fluorescence and luminescence, are generally used because of their sensitivity, which allows scaling down, such as to a 1536-well format. Miniaturization reduces the effect of light absorption but does not reduce interference caused by quenching of the excited state of the reporter by the test sample, so that light is not emitted" [61]. "In an assay in which the activity is measured by an increase in fluorescence intensity or luminescence, the test sample that quenches the signal causes interference with the readout measurement and results in showing a greater potency. Conversely, in an assay in which the activity is measured by a decrease in fluorescence intensity or luminescence, quenching by the test sample makes it to appear to have lower potency" [61].

6.1.3 Interference by test compounds which have fluorescence.

Some of the compounds in libraries have fluorescence of their own. The interference by such compounds results in misleading measurement of the assay and false positives are detected in such cases.

6.1.4. Interference with detection agents.

In some assays, detection of the product is indirect. It requires a chemical reaction with different reagents. "Some of the examples are the detection of free thiols by Ellman's reagent to produce a yellow product" [62] , the detection of inorganic phosphate by the malachite green reagent to generate an absorbance increase, and "the chemiluminescent detection of adenosine triphosphate (ATP) by the luciferin/luciferase reaction" [63]. The test compounds can reduce the sensitivity of an assay by reacting with one of the product formed and thereby generating false signals.

6.2. Materials and Methods.

6.2.1. Correcting for interference for test compounds.

To correct the interference from the compounds identified as hits from malachite green assay, we measured the extent of compound interference separately using an artifact assay and then applied a mathematical correction to the activity assay measurements based on the measured interference. To perform this assay, a separate plate was prepared containing the same arrangement of test compounds for each well as used in the activity assay. The artifact assay plate also consisted of wells in which no test compound was added and that is the baseline (B1). "Because it was required to measure an increase or a decrease in the baseline signal due to the presence of the test compound, each well in the artifact assay plate with and without test compound should contain the substance responsible for generating the signal, generally the product of the reaction used in the assay" [61]. In this case, it was P_i that was released as the reaction is catalyzed. In the artifact assay plate, 10 µM final concentration of Pi was added to each well to generate the signal. The assay was performed using malachite green assay and same conditions were used as in HTS. The only difference between the artifact assay and the activity assay was addition of P_i in place of the enzyme PurA.

6.2.2. Calculations for interference by test compounds

The absorbance at 623 nm was noted for the artifact assay plate. The interference was corrected according to the calculations in TABLE V.

TABLE V. CALCULATIONS FOR INTERFERENCE OF TEST COMPOUNDS IN THE ARTIFACT ASSAY

Artifact assay **Plate observation**	**Calculation**	**Activity assay** **Plate correction**
S1>B1	S1-B1	S2-(S1-B1)
S1<B1	S1/B1	S2 X B1/S1
S1>2 X B1	None	Reject data
S1< 0.5 X B1	None	Reject data

Where B1 = Baseline in artifact assay plate

S1 = Signal in test well of artifact assay plate

S2 = Signal in test well of activity assay plate.

"Data were rejected if the interference results in more than a 2-fold change in signal" [61].

6.3 Results and Discussion.

The shortlisted 13 compounds from malachite green assay hit validation were tested using the artifact assay. Out of 13 compounds, ten compounds were observed to be interfering with the assay and were false positive. Nine compounds including the 8 compounds of the same scaffold were rejected as they were interfering more than by the factor of 2 in the artifact assay. In order to confirm if these compounds were indeed false positives we proceeded to test them in a secondary independent binding assay.

CHAPTER 7: Binding analysis for compounds from Malachite green artifact assay

7.1. A Fluorescence Assay to determine ligand binding affinity

Proteins contain three aromatic amino acid residues (tyrosine, phenylalanine, and tryptophan) which lead to their intrinsic fluorescence. Out of the three, tryptophan has much intense fluorescence and higher quantum yield than the other two aromatic amino acids. Tryptophan shows fluorescence at excitation wavelength 280 nm and with maximum at 330–360 nm. The microenvironment of the tryptophan residues governs the intensity, quantum yield, and wavelength of maximum fluorescence emission of tryptophan. As the solvent polarity surrounding the tryptophan residue decreases, fluorescence spectrum shifts to shorter wavelength and the fluorescence intensity increases. Therefore, compared to tryptophan residues on the protein surface, tryptophan residues that are present in the hydrophobic core region of proteins dominate in the fluorescence emission intensity and have spectra shifted by 10 to 20 nm.

During protein-ligand interaction, quenching of fluorescence of the tryptophan residues of proteins by various inhibitors has been studied to confirm the binding site and to study the nature of the microenvironment of the tryptophan residues [64, 65]. Crystal structure analysis can be used to determine if there are several tryptophan residues near the catalytic site of the enzyme, and can notice changes in fluorescence with the binding of substrates or inhibitors. Thus, an easy and fast assay to analyze the binding of inhibitors to the catalytic site of the enzyme that alter tryptophan fluorescence could be developed and optimized.

7.2. Active Site Tryptophan of PurA

The crystal structure of PurA was analyzed to check whether there are tryptophan residues close to the catalytic site of PurA. Crystal structure of PurA from *Bacillus anthracis* str. Ames Ancestor (PDB: 4M0G) is a apo structure, so we aligned it with crystal structure of PurA from *Escherichia Coli* complexed with GDP, IMP, HADACIDIN, NO^{3-}, AND Mg^{2+} (PDB: 1GIN) to know about the proximity of tryptophan residues to the active site using UCSF Chimera. Three Tryptophan residues were found close to the catalytic site of PurA as shown in Figure 29.

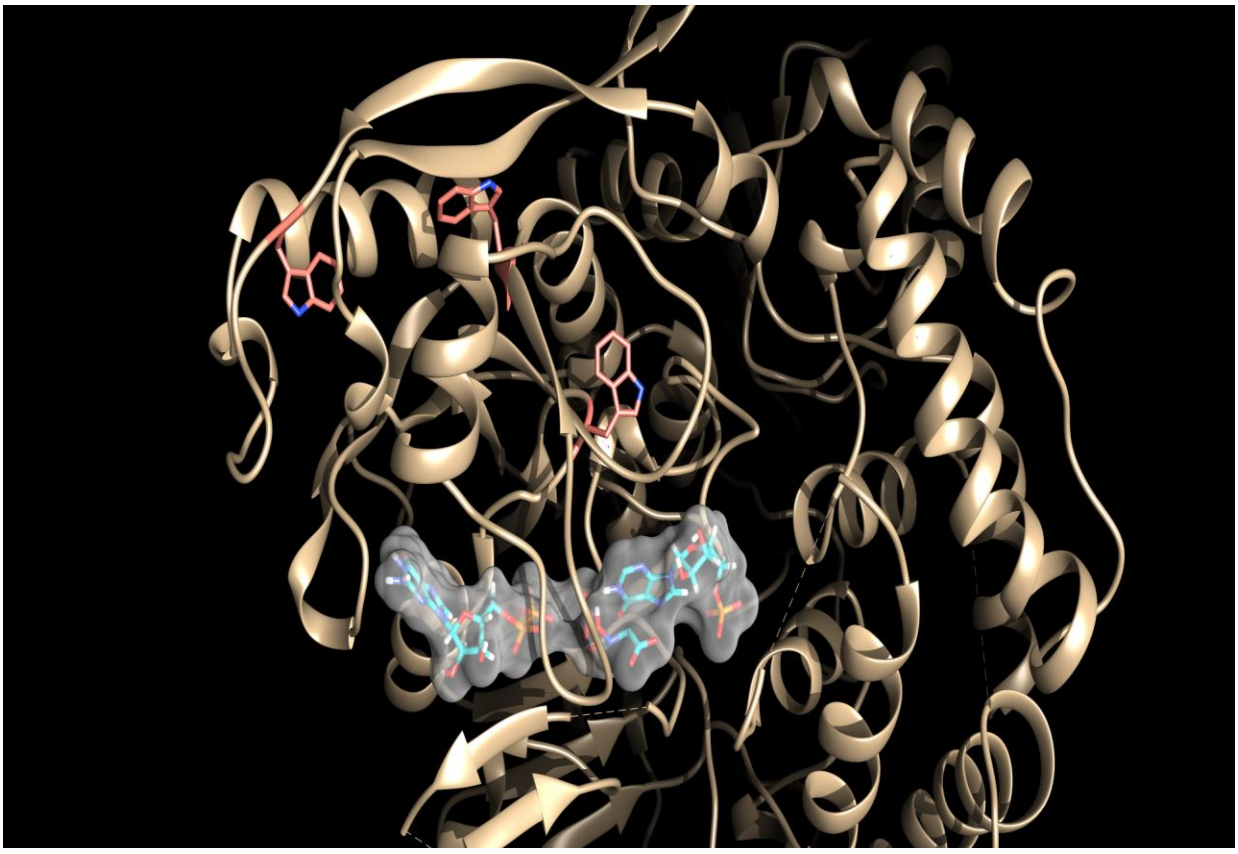


Figure 29. Structure of PurA shows presence of three tryptophan residues close to the catalytic site of the enzyme. The three-tryptophan residues are shown in pink color and the active site is shown as an envelope in gray color.

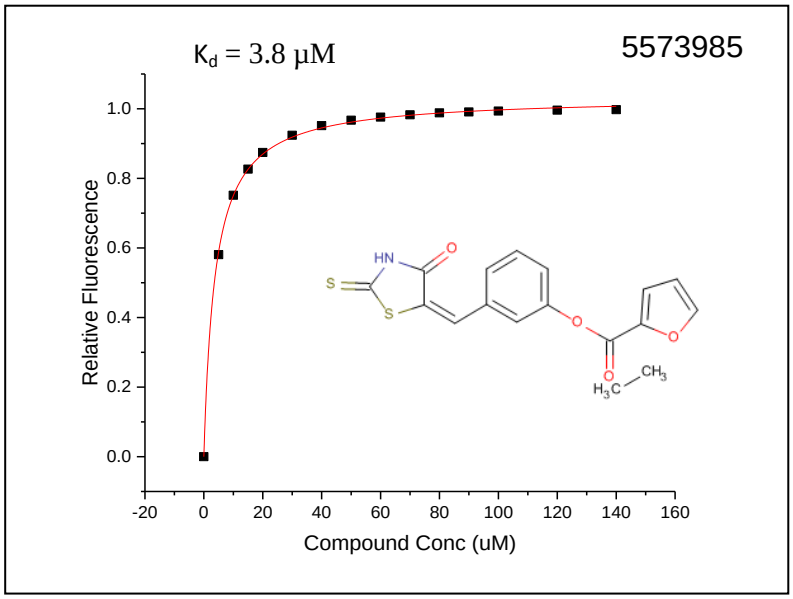
7.3. Materials and Method

Fluorescence measurements were done in a Varian standard 1 cm cuvette using the Varian Cary Eclipse Fluorescence Spectrophotometer at room temperature. Assay buffer consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 3 μM of purA and pure water. The final volume used for fluorescence measurements was 400 μl. PurA fluorescence spectra were taken at excitation 278 nm, and the emission was recorded between 300-450 nm (5 nm slit width, 'fast' scan mode). The concentration range of inhibitor tested was from 0 to 160 μM. The fluorescent intensities were corrected for the background fluorescence of compound. The inhibitor concentration on X-axis was plotted against the relative fluorescence on Y-axis, $(F_0\text{-}F)/F_0$ where F_0 is the maximum fluorescence (enzyme only) and F is the fluorescence at different inhibitor concentration.

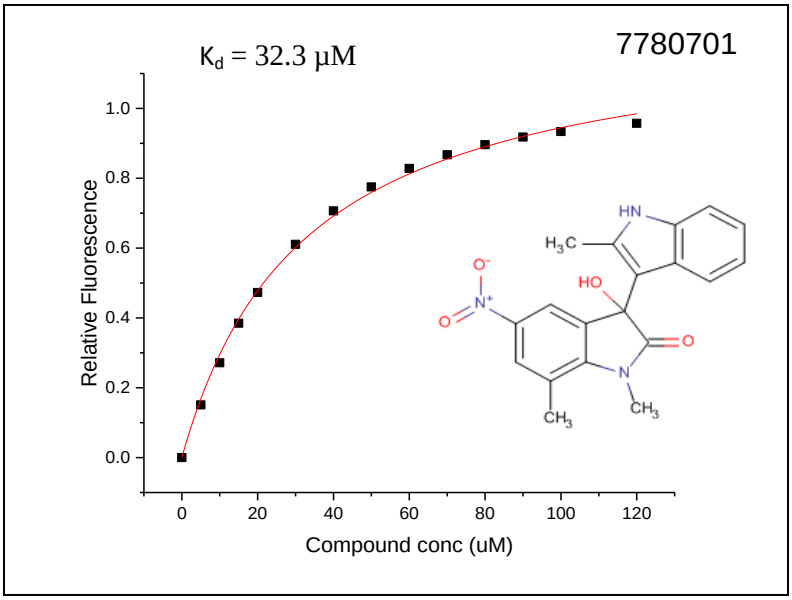
7.4 Result and Discussion

To confirm the results from artifact assay, four compounds, which had the same scaffold and highest % inhibition from HTS, along with four other compounds that passed in the artifact assay, were chosen and tested for their binding to the active site of the enzyme and dissociation equilibrium constant K_d was determined. Figure 30(A-H) shows the binding curve for these compounds. Relative fluorescence is on Y-axis and compound concentration on X-axis. The compounds that were found to be interfering with malachite green assay in the artifact assay showed weak binding to the active site of the enzyme and thus are false positives. Two compounds F1040-0011 and 5573985 were found to be promising hits with high % inhibition in malachite green assay and with K_d as 15.3 μM and 3.8 μM respectively. TABLE VI summaries the compounds from the primary screen analysis and the binding assay.

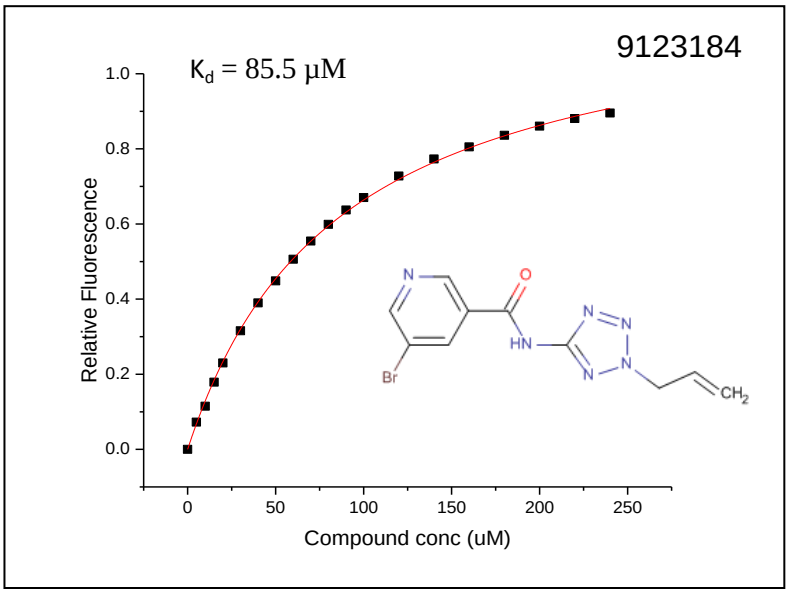
A


K_d = 3.8 µM
5573985
Relative Fluorescence
Compound Conc (uM)

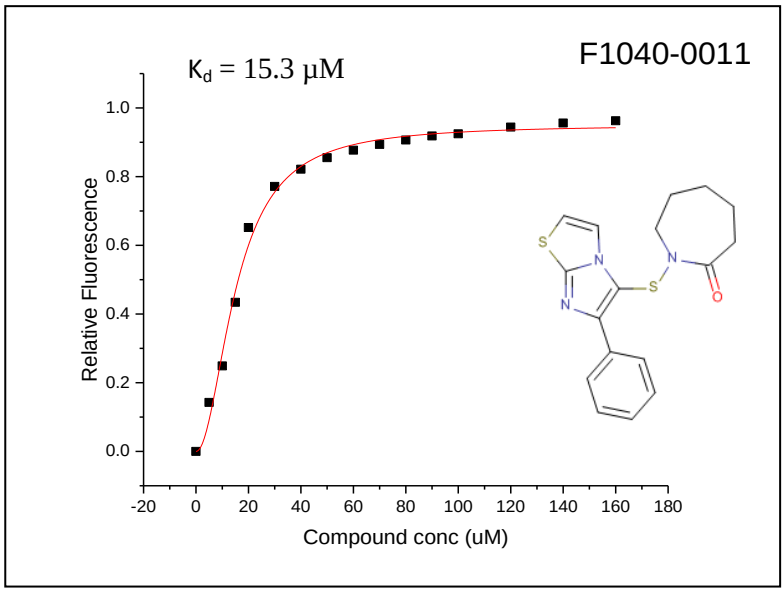
B


K_d = 32.3 µM
7780701
Relative Fluorescence
Compound conc (uM)

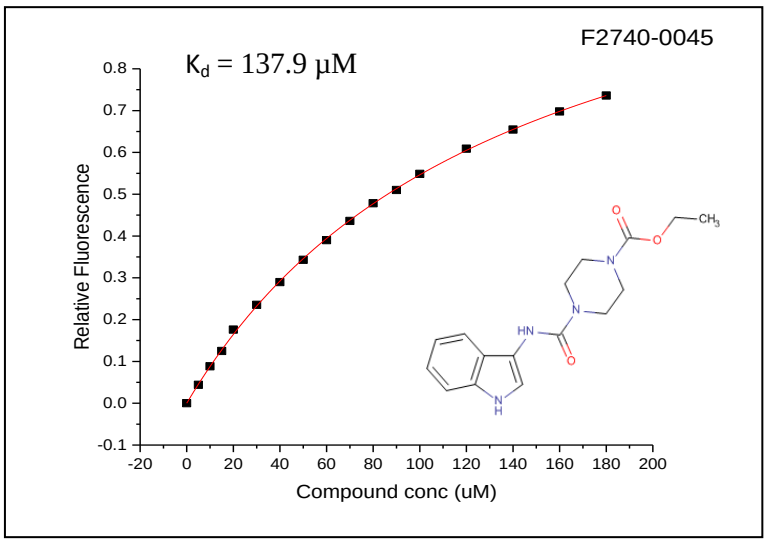
C


K_d = 85.5 µM
9123184
Relative Fluorescence
Compound conc (uM)

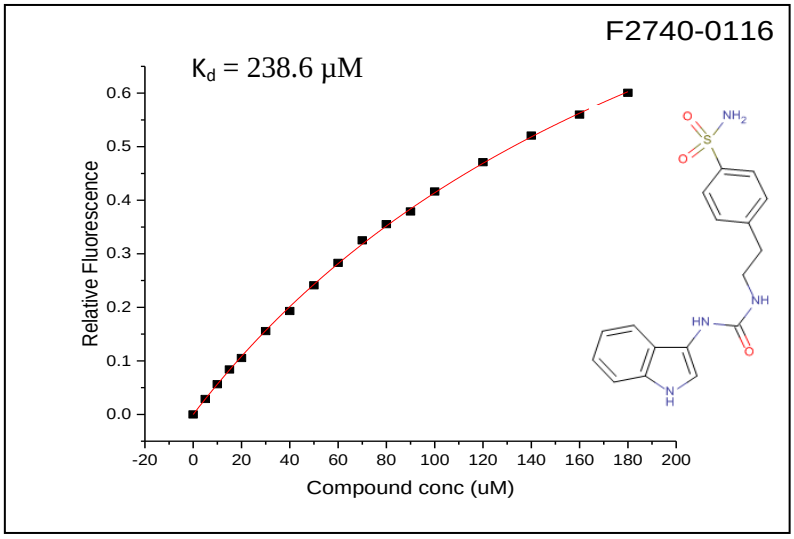
D


K_d = 15.3 µM
F1040-0011
Relative Fluorescence
Compound conc (uM)

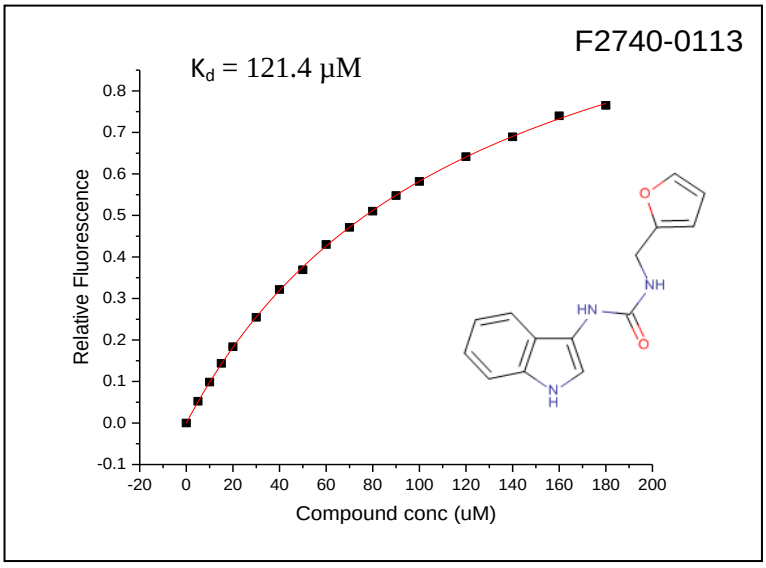
E



F



G



H

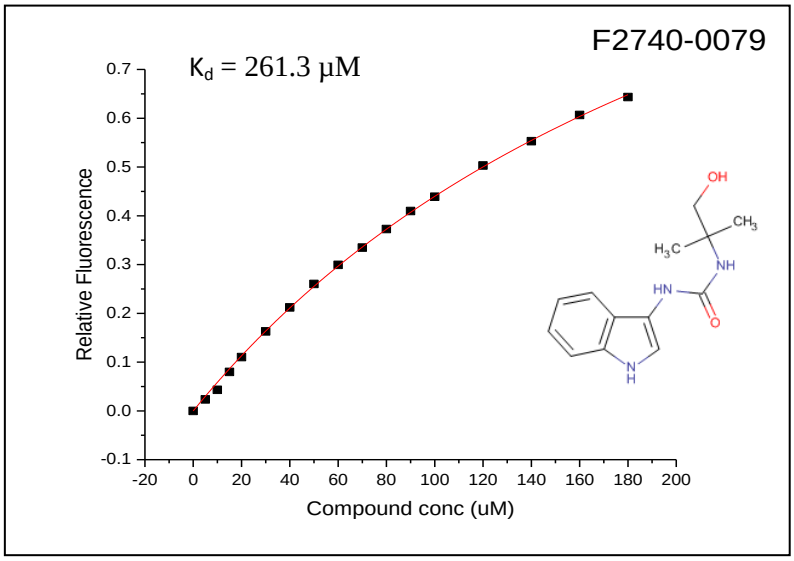


Figure 30(A-H). Binding curve for 8 compounds to be confirmed from artifact assay. (A) Compound 5573985 (B) Compound7780701 (C) Compound 9123184 (D) Compound F1040-0011 (E) Compound F2740-0045 (F) Compound F2740-0116 (G) Compound F2740-0113 (G) Compound F2740-0079.

TABLE VI. SUMMARY OF THE PRIMARY SCREEN AND BINDING ASSAY RESULTS OF THE COMPOUNDS

Compound	Mol Wt.	% Inhibition HTS at 29 µM	% Inhibition Run 2 at 29 µM	MIC µg/mL	K_d µM
5573985	331	59	38	12.5	3.8
7780701	351	37	42	>12.5	32.3
9123184	309	41	44	>12.5	85.5
F1040-0011	345	73	56	2.34	15.3
F2740-0116	358	61	39	>12.5	238.6
F2740-0113	255	61	43	>12.5	121.4
F2740-0045	316	61	49	>12.5	137.9
F2740-0079	247	62	49	>12.5	261.3

CONCLUSION

Bacillus anthracis causes an acute infectious disease called anthrax. It is classified as a Category A agent pathogen. The standard treatment for anthrax is use of antibiotics such as tetracycline, ciprofloxacin or doxycycline. However, resistant *B. anthracis* strains have been reported. Thus, it is necessary to develop novel therapeutic agents against *B. anthracis*.

Adenylosuccinate synthetase (PurA) is an important enzyme in the bacterial purine biosynthesis pathway and various knockout studies have shown that PurA is essential for survival of bacteria. In addition, it is found as a good target for druggability with a drugable score of 0.8.

In order to identify novel inhibitors against *B anthracis* PurA, malachite green assay was optimized and high-throughput screening was performed using the Life Chemicals and the Chembridge libraries with an ideal Z'-factor. The hits from malachite green assay were further analyzed for reproducibility, and interference with the assay using the artifact assay.

To further confirm the hits from artifact assay, an independent binding assay called tryptophan fluorescence assay was employed. Two compounds were found to have good binding affinity to the active site of the enzyme.

In summary, we have identified two compounds that appear to be promising hits for further development in a hit-to-lead optimization process. Both these compounds have good inhibition in HTS, a good MIC and bind to the enzyme. Further, a secondary assay would be optimized to determine the IC_{50} for these compounds that would tell the amount of inhibitor required to inhibit the PurA reaction. After determining the IC_{50}, these hits would be subjected to structure activity relationship (SAR) to study the relationship between the inhibitor molecular structure and its

biological activity. An SAR study would enable us to determine the chemical groups responsible for evoking a target biological response in the organism. Structure activity analysis would help in designing drugs with greater potency and fewer side effects. Once SAR analysis is performed, we will use X-ray crystallography to visualize the inhibitor-protein co-crystal structures at atomic level and will help in understanding the function and interaction of the inhibitor with the residues of PurA. After confirming these hits as leads, they would be further developed in drug discovery process.

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3. "Development of inhibitors against *S.pneumoniae* SAICAR synthase (PurC)" presented at Research Day held at College of Pharmacy, University of Illinois at Chicago, March 2012.

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