PROSPECTS OF INDOLE DERIVATIVES AS METHYL TRANSFER INHIBITORS: AMR MANAGERS

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LIST OF ABBREVIATIONS

AA	Amino Acid
ABC	ATP Binding Cassette
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
AMEs	Aminoglycoside Modifying Enzymes
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BE	Binding Energy
CATs	Chloramphenicol Acetyltransferases
CDC	Centers for Disease Control and Prevention
cFID	Consensus Induced-Fit Docking
CLEVER	Chemical Library Editing, Visualizing and Enumerating Resource
COBRA	Constraint-based Reconstruction and Analysis
CSDD	Center for the Study of Drug Development
DNA	Deoxyribonucleic Acid
ED	Ensemble Docking
FBA	Flux Balance Analysis
FDA	Food and Drug Administration
FMN	Flavin Mononucleotide
Gbk	Gene Bank
HGT	Horizontal Gene Transfer
HTS	High Throughput Sequencing
ISP	International *Streptomyces* Project
LBVS	Ligand-Based Virtual Screening
LCBs	Locally Collinear Blocks
MATE	Multidrug And Toxic compound Extrusion
MD	Molecular Dynamics
MDR	Multi-Drug Resistance
MFS	Major Facilitator Superfamily
MGE	Mobile Genetic Elements
MLS	Macrolide-Lincosamide-StreptograminB
NCBI	National Center for Biotechnology Information
PAINS	Pan Assay Interference Compounds
PBP	Penicillin Binding protein
PDB	Protein Data Bank
PG	Peptidoglycan
PKC	Proteinase Kinase C
PSA	Polar Surface Area
RBS	Ribosome Binding Site
RNA	Ribonucleic acid
RND	Resistance-Nodulation-Division family

SAM	S-Adenosyl Methionine
SBVS	Structure Based Virtual Screening
SD	Shine-Dalgarno Sequence
SEA	Similarity Ensemble Approach
SMR	Small Multidrug Resistance
Tm	Melting temperature
TMP	Trimethoprim
Tox	Toxicity
TPP	Thiamine pyrophosphate
tPSA	Topological Polar Surface Area
TSS	Transcription Start Site
UTR	Untranslated region
VS	Virtual Screening
WHO	World Health Organization

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ABSTRACT

It is prudent that new antibiotics be urgently developed as to manage WHO listed critical and high priority nine multi-drug resistant (MDR) pathogens posing unprecedented medical crisis. Simultaneously, multiple essential proteins have to be targeted to prevent easy resistance development. Whole genome sequences of these pathogens were aligned exploiting DNA alignment potential of MAUVE software to identify putative common lead target proteins in all nine pathogens. S-adenosyl methionine (SAM), a critical metabolite in several biochemical reactions, biosynthesizing *metK* gene was taken as the lead target and the gene essentiality analysis using COBRA tool revealed that SAM is a critical metabolite. Furthermore, gene essentiality of corrin methylation steps revealed *cobA* gene as essential. Hence, from the library of 715 indole derivatives, chosen based on kinase inhibition potential of some indoles, around 102 were pursued based on ADME/T scores. Among these, the 58 higher binding derivatives against *N. gonorrhoea* MetK were further expanded to MetK proteins of all nine pathogens and 9 derivatives exhibited higher binding energy. These, 58 upon docking to other SAM utilizing enzymes, CobA, CysG, Dam, TrmD and *cis* regulatory RNA SAM-I riboswitch, 8 derivatives had higher binding energy and six had multi-target including MetK in all pathogens. Further, docking with human metK homologue showed 1-Methyl-3,4-bis(3-indolyl) maleimide as the only drug candidate with minimal effects on human but inhibitory effects on all studied targets. The molecule could be developed to treat infections caused by *N. gonorrhoea, A. baumanii, C. coli, K. pneumoniae, E. faecium, H. pylori, P. aeruginosa, S. aureus* and *S. typhii*.

Key words: *In silico* virtual screening, WHO priority pathogens, drug resistance, putative antimicrobials, Indole

1. INTRODUCTION

1.1 Background

The finding and development of antibiotics was a milestone in medical sciences that prevented fatality from simple infections. Unfortunately, the emergence of antibiotic resistant strains among these pathogens appear to be inevitable as selective pressure for survival (Tello *et. al.*, 2012). Such resistances are developed due to, partly because of the reduced patient compliance (Malfertheiner, 1993), the mutations in bacteria (Woodford and Ellington, 2007) and adaptability through horizontal gene transfer (Burmeister, 2015). The most alarming is the prevalence of resistance even in the last resort antibiotic colistin (Caniaux *et. al.*, 2017) that has added serious challenge to the current antibiotic crisis.

New antimicrobials are sought against the highly prioritized pathogens published by WHO in 2017 as Critical and High priority (Lawe-Davies and Bennett, 2017). However, new antibiotics have not yet been discovered and developed for human application for almost two decades. Virtually all of the easily discovered novel antibiotics were found in between 1929 and 1962. In the intervening period, analogues of old compounds were developed. However, the number of chemical modifications that can be made to an old compound, for example penicillin, are finite. Thus, new clinically important analogues to old antibiotics that could be active against resistant pathogens would become more difficult to make as the options are choked. And only two novel classes of antibiotics, the oxazolidinones (Barbachyn and Ford, 2003) and the cyclic lipopeptides (Kern, 2006) have entered the market since 2000.

This is why discovery of novel families of antibiotics at a regular interval is a must if modern medicine is to continue in its present form. In an ecologically diverse country like Nepal - 27th in the whole world (Butler, 2016), there are many yet to be explored places which can be excellent habitats for *Streptomyces* strains that could have the ability to produce noble Antibiotics, eventually helping in coping the present diseases and antibiotic resistance problems. In addition, use of computational biology approach (March-Vila *et. al.*, 2017) could help in identifying noble lead target proteins against which library of chemicals could be virtually screened to narrow down starting molecule which could be then incrementally modified for development.

1.2 Current Studies

Hit to lead identification has been the most preferred drug discovery process (Keseru and Makara, 2006) which involves small molecule hits from a high throughput screen (HTS) and developing these as promising lead compounds. Medicinal chemistry has also

been an important aspect of modern drug discovery (Vemuri and Makriyannis, 2015) which involves design and chemical synthesis of pharmaceutical agents.

In 2003, only 35 new compounds were registered with the Food and Drug Administration (FDA) with a research expense of approximately 33 billion dollars (Spedding, 2006). Similarly, the cost of developing a prescription drug estimated by The Tufts Center for the Study of Drug Development as published in the Journal of Health Economics in March 2016, is a massive 2.558 billion dollars (TUFTS CSDD R&D Cost Study, 2016). The rapid increase in drug resistance among pathogens and the excessive time and cost parameters required to develop a drug, demand a robust and faster method of drug discovery. The computational strategies come into play, assisting drug discovery and development in an efficient manner with the available *in vitro* techniques (Sliwoski *et al.*, 2014).

Despite search for new antibiotics, computational approach could be taken to screen the library molecules available in different databases. However, for such screens lead protein identification is required to develop the drug that acts against the protein. Moreover, the lead protein should be essential for survival of the pathogen and the molecule should be able to act against multiple targets so that the pathogen would not develop resistance against it easily.

In this research, the works are solely based on application of various computational techniques to ease the drug development process and possibly prevent the developed lead molecules from being unsuccessful in the later stages of drug development including various phases of pre-clinical trials.

1.3 Hypothesis

1.3.1 Null hypothesis:

Potential lead molecules will not be developed against the resistant pathogens.

1.3.2 Alternative hypothesis:

Potential lead molecules will be developed against the resistant pathogens.

1.4 Objectives

1.4.1 General Objective

- • To identify novel lead molecules against the resistant pathogens prioritized by WHO for new drug development

1.4.2 Specific Objectives

- To identify probable drug targets
- To create a ligand database for virtual screening

- To prepare 3D protein structures using online tools
- To perform molecular docking against the drug targets
- To look for lead molecules that could potentially be developed as drugs with minimal toxicity to humans.

The development of novel antibiotics took a serious downfall since the mid-19th century. However, the emerging resistance among the deadly pathogens demands newer antibiotics to be developed. Huge research cost and numerous failures in various stages of drug development have lowered the interests of commercial pharmaceutical companies in such investments. The present study is focused on developing a robust method for target identification, lead identification through virtual screening and developing a probable mechanism of inhibition study method for potential lead molecules against a drug target.

1.6 Scope of study

The present study focuses on identifying potential lead molecules among the Indole derivatives against multiple drug targets in emerging pathogens. Also, robust screening and antimicrobial assays have been sought.

2. LITERATURE REVIEW

2.1 Review of literature related to antimicrobial resistance and the underlying mechanisms

2.1.1 Antimicrobial resistance – a global concern

WHO defines the antimicrobial resistance (AMR) as the state of a microorganism (bacteria, viruses, and some parasites) that is involved in communicable disease and it's transmission, when it garners the ability to prevent an antimicrobial (such as antibiotics, antivirals and antimalarials) from exhibiting intended detrimental effect against these microbes making the standard treatments ineffective and retaining the probability of communicability of infections (http://www.who.int/antimicrobial-resistance/en/). Thus, AMR and especially multi-drug resistance (MDR) where the bugs exhibit resistance against three or more antimicrobials is of serious global concern.

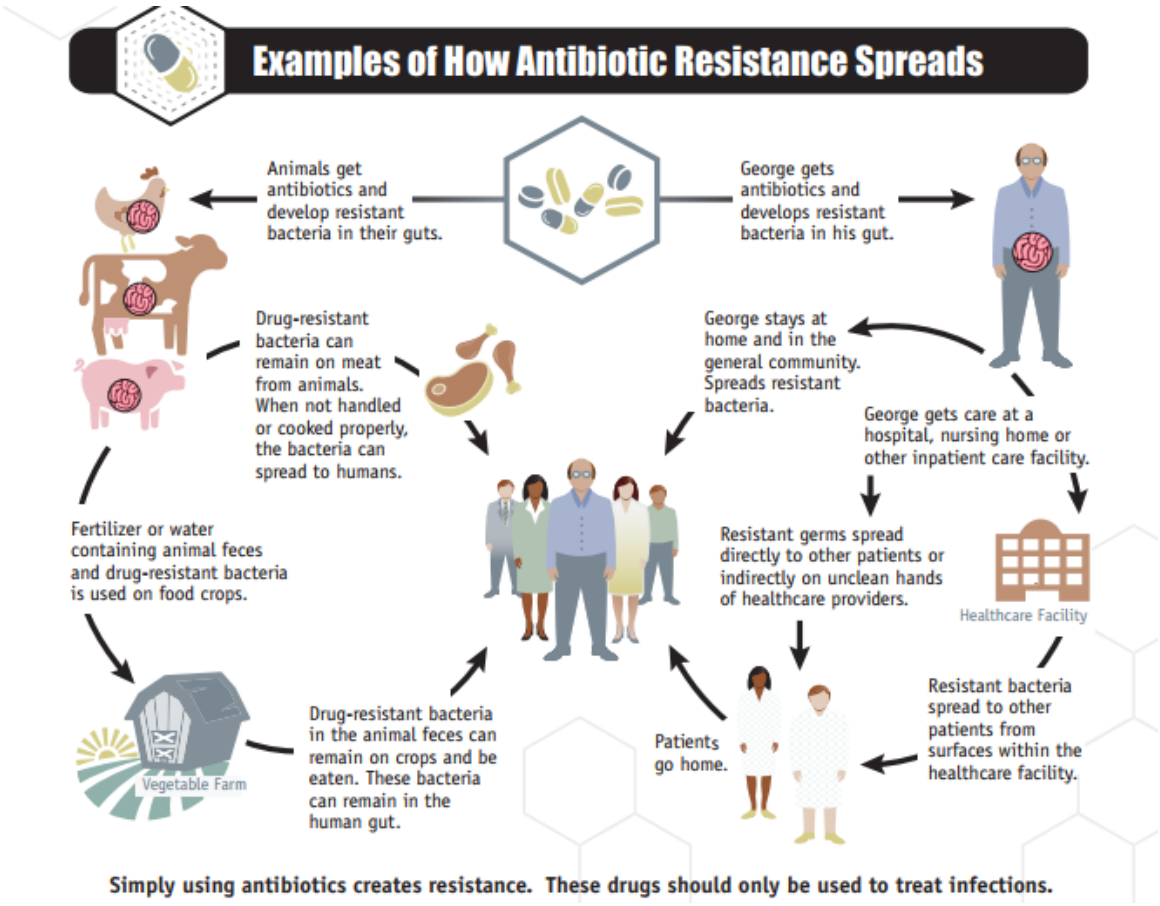


Figure 1: A simple illustration of how antibiotics spread among different hosts (CDC, 2013).

Antibiotic resistance has been named as one of the three most important public health threats for the 21st century (World Health Organization, 2014). This could be correlated by the fact that even in the United States alone, at least 2 million people acquire serious antibiotic-resistant infections and around 23,000 respective fatalities each year, (Centers

for Disease Control and Prevention – CDC). Due to the mobility and travel of human, new forms of antibiotic resistance can cross international boundaries with ease and on a remarkable speed. Due to diverse hosts of these pathogens (Bowden and Drake, 2013) antibiotic resistant microorganisms have been described as "nightmare bacteria" that "pose a catastrophic threat" to all living beings in every nooks and corners of the world, either developed or least developed countries.

The new resistance mechanisms are emerging and spreading all around the globe in an unprecedented way (Ferri *et al.*, 2015). This is threatening the ability to treat even the common infectious diseases that were manageable till recent past and now is resulting in prolonged illness and in some cases death, also. Overuse (Shallcross, 2014), inappropriate antibiotic selection (Shapiro *et al.*, 2014), unnecessary antibiotic prescriptions (Fleming-Dutra *et al.,* 2016) and lack of patient compliance are thought to be the reasons for this emergence of resistance.

Hence, drug or antimicrobial resistance appears to be the major complications for managing infections (Llor and Bjerrum, 2014). In addition, heteroresistance (presence of minor sub-population of resistant bacteria in an otherwise susceptible bacterial culture) to colistin (Loho and Dharmayanti, 2015), a last resort antibiotic observed among highly resistant (including carbapenem and β-lactams resistant) *Klebsiella pneumoniae* isolates (Halaby *et al.*, 2016), has added up the burden of finding alternative treatment than the present antibiotics world.

2.1.2 Mechanisms of Antibiotic Resistance

2.1.2.1 Genetic Basis of Antimicrobial Resistance

Genetic plasticity allows bacteria to survive a wide range of environmental threats including detrimental effect of antimicrobials. From an evolutionary perspective of survival, bacteria have been found to execute two major genetic strategies to adapt in the presence of antimicrobials in its ecological niche; i) mutation in gene(s) associated with the target protein against which the mechanism of action of the antimicrobial compounds are used for and ii) horizontal gene transfer (HGT) of foreign DNA coding for resistance (Munita and Arias, 2016).

These genetic alterations mainly change the antibiotic action via one of the following mechanisms; i) modifications of antimicrobial target, ii) decrease in drug uptake, iii) increase in efflux, and iv) global changes in important metabolic pathways (described in later sections).

Horizontal Gene Transfer (HGT)

Transfer of antibiotic resistant genes among bacteria (Harris *et. al.*, 2010) could be achieved through HGT, the movement of genetic information between organisms,

fueling pathogen evolution (Burmeister, 2015) that may result in bacteria developing greater resistance (Stanczak-Mrozek *et. al.*, 2015).

Generally, bacteria acquire external genetic materials through three main strategies; i) Transformation (incorporation of naked DNA), ii) Transduction (phage mediated), and iii) Conjugation. Conjugation involves cell-to-cell contact, prevalent mostly in the gastrointestinal tract of humans under antibiotic treatment and is the most extensive HGT in the medical centers developing resistance among the pathogens (Waters, 1999).

Despite the fact that a number of processes limit the HGT including Surface exclusion, Restriction endonucleases cutting down the foreign genetic materials, barriers to plasmid replication and establishment in a heterologous host (Thomas and Nielsen, 2005) but some pathogens appear to overcome these limitations for developing resistance for survival and evolution (Andam *et. al.*, 2011).

In conjugation, although direct transfer from chromosome to chromosome has also been observed (Manson *et. al.*, 2010) but generally uses mobile genetic elements (MGEs) mostly plasmids and transposons as vehicles (Rankin *et. al.*, 2011) to share valuable genetic information beneficial for those hosts including antimicrobial resistance trait.

In addition to MGE, Integrons, are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes (Hall and Collis, 1995). Integrons seem to have a major role in the spread of multidrug resistances among bacteria (Ploy *et. al.*, 2000). Most probably, through non-homologous joint recombination (Vries and Wackernagel, 2002) regardless of chromosomal loci or through homologous recombination (Domingues *et. al.*, 2012) where the antibiotics resistance gene is flanked by the common sequences in both organisms. These contribute to the addition of new genes into the bacterial chromosomes, and also ensure their expression.

2.1.2.2 Mechanistic Basis of Antimicrobial Resistance

A bacterial cell may develop resistance against a particular class of antimicrobials via a number of mechanisms through multiple biochemical pathways. The three fundamental mechanisms of antimicrobial resistance include i) Changes in membrane permeability to antibiotics ii) enzymatic inactivation of antibacterial drugs and iii) alteration of bacterial target proteins/pathways (Dever and Dermody, 1991).

2.1.2.2.1 Decreased Antibiotic uptake and Efflux

2.1.2.2.1.1 Decreased permeability

Hydrophilic antibiotics including β-lactams, tetracyclines and some fluoroquinolones use porins to cross membrane barrier (Pagès *et. al.*, 2008) and hydrophobic antibiotics including some aminoglycosides, macrolides, rifamycins and cationic peptides are

transported through the outer membrane bilayer (Delcour, 2009), so all of these are affected by changes in the permeability of the outer membrane. Bacteria have developed mechanisms to stop the antibiotics from reaching their cellular target by decreasing uptake of the antimicrobials (Miller *et. al.*, 2014), which is a major issue since most antibiotics currently in use have intracellular targets located inside inner membrane in case of gram negative bacteria. .

2.1.2.2.1.2 *Efflux pumps*

In addition, some pathogens develop ability to flush out the antibiotics even if they cross the membrane barrier by efflux pumps that pump solutes out of the cell. Localized in the cytoplasmic membrane of all kinds of cells, efflux pumps are proteinaceous transporters that can extrude a wide range of substrates including antibiotics. There are five major families of efflux transporters; the major facilitator superfamily (MFS) (Pao *et. al.*, 1998), the small multidrug resistance (SMR) family (Chung and Saier, 2001), the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Choi, 2005), the multidrug and toxic compound extrusion (MATE) family (Kuroda and Tsuchiya, 2009) and the resistance-nodulation-division (RND) family (Fernando and Kumar, 2013).

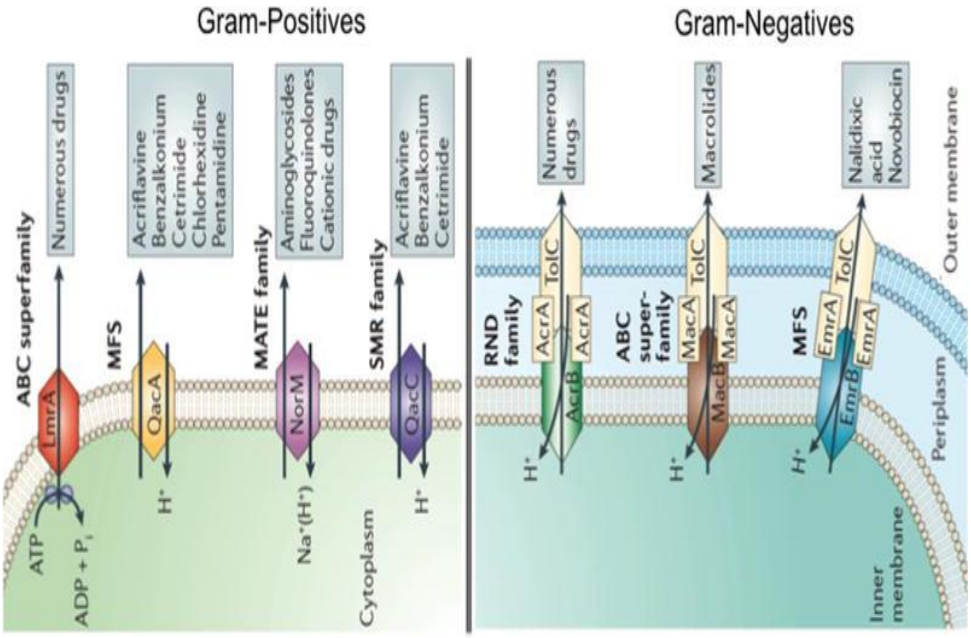


Figure 2: Representation of five different types of efflux pumps in gram-positive and gram negative bacteria (Munita and Arias, 2016).

2.1.2.2.2 Modification of the Antibiotic molecule

One of the most successful bacterial strategies that bring about antimicrobial resistance is the production of certain enzymes that inactivate the antibiotics or that destroy these molecules itself.

Modification of molecules as antibiotic resistance could be acquired in both gram positive and gram negative bacteria by the production of enzymes, capable of altering the antibiotic molecules by various mechanisms including hydrolysis, group transfer and redox mechanisms. Group transfer approaches are the most diverse including modification of antibiotics by acyltransfer, phosphorylation, glucosylation, nucleotidylation, ribosylation and thiol transfer (Wright, 2005). The respective modifications are described below.

2.1.2.2.2.1 Hydrolysis

Many antibiotics contain esters and amides having hydrolytically susceptible bonds that are cleaved by enzymes and several such enzymes have evolved. They subsequently destroy the antibiotic activity. The most common include amidases that cleave the β-lactam ring of Penicillin and Cephalosporin classes of antibiotics (Frère, 1995). Others include esterases linked to macrolide resistance (Nakajima, 1999) and epoxidases linked to fosfomycin resistance (Castañeda-García *et. al.*, 2013). These enzymes solely require water as the co-substrate and are often produced by resistant bacteria thereby intercepting antibiotics before their contact with bacterial target.

2.1.2.2.2.2 Group Transfer

The group transfer family of enzymes covalently modify antibiotics resulting in the structural changes that impair target binding potential. Chloramphenicol acetyltransferases (CATs) modify Chloramphenicol, a broad spectrum antibiotic used against numerous infections (Shaw, 1983). In addition, clinically significant examples of drug modifications via group transfer mechanisms include aminoglycoside modifying enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the aminoglycosides (Ramirez and Tolmasky, 2010)

2.1.2.2.2.3 Others

Beyond enzymatic hydrolysis and group transfer resistance mechanisms, alternative strategies to engender resistance have been growing consistently in bacteria. These include Redox enzymes and Lyases. Oxidation-reduction of antibiotics have not been frequently exploited by bacteria. The few illustrations include oxidation of tetracyclines by TetX enzyme, reduction of a critical ketone group to alcohol at 16^{th} position by *Streptomyces virginiae* to protect against its own antibiotic Virginiamycin M1 (Speer and Salyers, 1988; Lee *et. al.*, 1996). Similarly, Lyases are C-C, C-O, C-N, and C-S cleaving enzymes by non-hydrolytic and non-oxidative processes. An antibiotic resistance lyase, VgB is responsible for type B streptogramin resistance (Mukhtar *et. al.*, 2001).

Table 1: Mode of inactivating antibiotics by resistant organisms (Wright, 2005)

Strategy	**Type**	**Antibiotics affected**
Hydrolysis		β-lactams
		Macrolides
Group transfer	Acyl	Aminoglycoside
		Chloramphenicol
		Type A Streptogramin
	Phosphoryl	Aminoglycoside
		Macrolide
		Rifamycin
		Peptide
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycoside
		Lincosamide
	ADP-ribosyl	Rifamycin
	Glycosyl	Macrolide
		Rifamycin
Others	Redox	Tetracycline
		Rifamycin
	Lyase	Type A Streptogramin
		Type B Streptogramin

2.1.2.2.3 Changes in Target Sites

Another common strategy developed by bacteria against antibiotics is to interfere with their target sites. It includes target protection (avoiding contact of antibiotics and target sites) and target site modification (decreased affinity of target site receptors with antibiotics).

2.1.2.2.3.1 Target protection

The competitive inhibition of antibiotic binding sites with bacterial proteins prevents antibiotics from binding and thereby prevents its activity. One of the best studied target protection mechanisms is the tetracycline resistance determinants TetM and TetO, which compete binding with tetracycline for the same ribosomal space and in addition, alter the binding site as well (Donhofer *et. al.*, 2012; Li *et. al.*, 2013).

2.1.2.2.3.2 Modification of target site

This resistance mechanism affects almost all families of antimicrobial compounds. These modifications can be either i) point mutations in the genes encoding the target site, ii) enzymatic alterations of binding site or iii) replacement of the original target.

Rifampin resistance by single-step point mutations in rpoB gene (Aubry-Damon *et. al.*, 2002), macrolide, lincosamide and streptogramin B (MLSB) antibiotics resistance by methylation of a specific adenine residue in 23s rRNA catalyzed by enzymes coded by erythromycin ribosomal methylation (*erm*) genes (Maravić, 2004), linezolid resistance by mutations in genes *rpl*C and *rpl*D (Mendes *et. al.*, 2014) are some important examples.

Also, Bacteria are capable of evolving new targets such that they have similar biochemical functions as the original target but are unknown to the antibiotics. Methicillin resistance in *Staphylococcus aureus*, in addition to penicillin binding proteins (PBP), the acquiring of an exogenous PBP (PBP2a), is among the most relevant clinical example (Stapleton and Taylor, 2007).

Another strategy is by production of antimicrobial targets in bulk for overcrowding the antibiotics by the increased number of available targets. An important example is the resistance development against trimethoprim-sulfamethoxazole (TMP-SMX) due to the massive overproduction of the target enzyme, dihydrofolate reductase (Flensburg and Skold, 1987; Eliopoulos and Huovinen, 2001).

2.2 Review of literature related to current Antibiotics and their underlying mechanisms

More effective antimicrobials have been discovered and developed by modification of drug molecules since the discovery of the first antibiotic penicillin produced by a fungus *Penicillium chrysogenum* in 1929 (Ligon, 2004). The basic mechanisms of antibiotics activity include inhibition of cell wall synthesis (most common), inhibition of protein synthesis / translation (second largest class), cell membrane alteration, inhibition of nucleic acid bio-synthesis, and antimetabolite activity / competitive inhibition.

2.2.1 Inhibition of cell wall synthesis:

Bacteria are enclosed by peptidoglycan (PG) layers with the mechanical strength that is critical for a bacterium's ability to resist unfavorable environmental conditions. PG layers are maintained by the activities of transglycosylase and transpeptidase enzymes that function in linking of disaccharide pentapeptides to extend the glycan strands of existing PG molecules and in cross linkage of adjacent peptide strands of immature PG units, respectively (Park and Uehara, 2008). Those molecules that act on inhibition of biosynthesis are taken as good antibiotics and β-lactam ring containing compounds are group of antibiotics in this class. This results in bacterial death by lytic or non-lytic mechanisms depending upon bacteria and enzyme acted upon.

2.2.1.1 **Lytic cell death**

Certain classes of antibiotics such as β-lactams interfere with specific steps in cell wall biosynthesis resulting in changes in cell shape, size, induction of cellular stress and ultimately cell lysis (Tomasz, 1979). β-lactams including penicillins, carbapenems and cephalosporins inhibit transpeptidases activity. The β-lactam drug molecule with a cyclic amide ring, is a structural analogue of the terminal D-alanyl-D-alanine dipeptide of PG, and acts as a substrate for transpeptidases during the acylation phase of cross-link formation. The enzyme is unable to hydrolyze the bond created with the β-lactams thus disrupting the cell wall by inhibiting formation of PG layers (Williamson *et. al.*, 1986).

On the other hand, Glycopeptides (e.g. Vancomycin) inhibit PG synthesis by binding with PG units (at D-alanyl-D alanine dipeptide) and by blocking the activities of transglycosylases and transpeptidases.

2.2.1.2 Non-lytic cell death

Unlike cell lysis due to triggering of major autolytic enzyme (an N-acetylmuramoyl-L-alanine amidase), some β-lactams have been reported to kill the mutants without the amidase depicting the cell death with autolysis-independent mechanisms (Moreillon *et. al.*, 1990).

2.2.2 Inhibition of Protein synthesis:

The transcribed mRNA is decoded in a ribosome to produce a specific amino acid chain peptide linkage and later on this peptide folds into an active protein. Mainly, mRNA translation occurs in three sequential phases – initiation, elongation and termination. This involves the ribosome and various accessory factors. The ribosome organelle comprises two ribonucleoprotein subunits – the 50s and the 30s. These organize to become 70s unit upon the formation of complex between an mRNA transcript, various initiation factors, and a fMet-charged aminoacyl-tRNA (Nissen *et. al.*, 2000). Thus, the

antibiotics that act as protein synthesis inhibitors can be grouped as 50s and 30s ribosome subunit inhibitors.

2.2.2.1 50s ribosome subunit inhibitors

In general, these antibiotics block either the initiation of protein translation or the translocation of peptidyl-tRNAs resulting in inhibition of peptidyltransferase reaction responsible for the nascent peptide chain elongation. For example, the macrolide-lincosamide-streptogramin B (MLS) class of antibiotics illustrate similar function in binding to the 50S ribosomal subunit and inhibiting protein biosynthesis by dissociating the peptidyl-tRNA from ribosome and/or blocking the exit path of the nascent peptides from the ribosome (Tenson *et. al.*, 2003). Similarly, though different than MLS, oxazolidinones bind at the P site at ribosomal 50S subunit and inhibits protein synthesis (Bozdogan and Appelbaum, 2004).

2.2.2.2 30s ribosome subunit inhibitors

In prokaryotes, 30S subunit, the smaller subunit of the 70S ribosome is a complex of 16s ribosomal RNA and 19 different proteins (Schluenzen *et. al.*, 2000) and mainly functions to initiate translation at the correct location by recognizing the Shine-Dalgarno sequence (Omotajo *et. al.*, 2015). The 30S ribosome inhibitors include mainly aminoglycosides families of antibiotics, tetracyclines and aminocyclitol class comprising spectinomycins. Aminoglycosides interact with 16s rRNA to cause conformational changes in the complex between mRNA and tRNA at the ribosome thus resulting protein mistranslation (Kotra *et. al.*, 2000), tetracycline blocks the access of aminoacyl-tRNAs to the ribosome (Chopra and Roberts, 2001), and spectinomycins interfere with the stability of peptidyl-tRNA binding to ribosome (Borovinskaya *et. al.*, 2007).

2.2.3 Inhibition of Nucleic Acid Synthesis

2.2.3.1 Inhibition of DNA replication

Quinolones interfere with the maintenance of chromosomal topology by targeting DNA gyrase enzyme responsible for supercoiling of DNA and topoisomerase IV by trapping these enzymes at the DNA cleavage stage and by preventing the rejoining of the strands (Chen *et. al.*, 1996).

2.2.3.2 Inhibition of RNA synthesis

A semi-synthetic class of bactericidal antibiotics, rifamycins inhibit RNA synthesis by binding DNA dependent RNA polymerase with a strong affinity and blocking the initiation process thus is a potent means of bacterial cell death (Calvori *et. al.*, 1965).

2.2.4 Antimetabolite activity

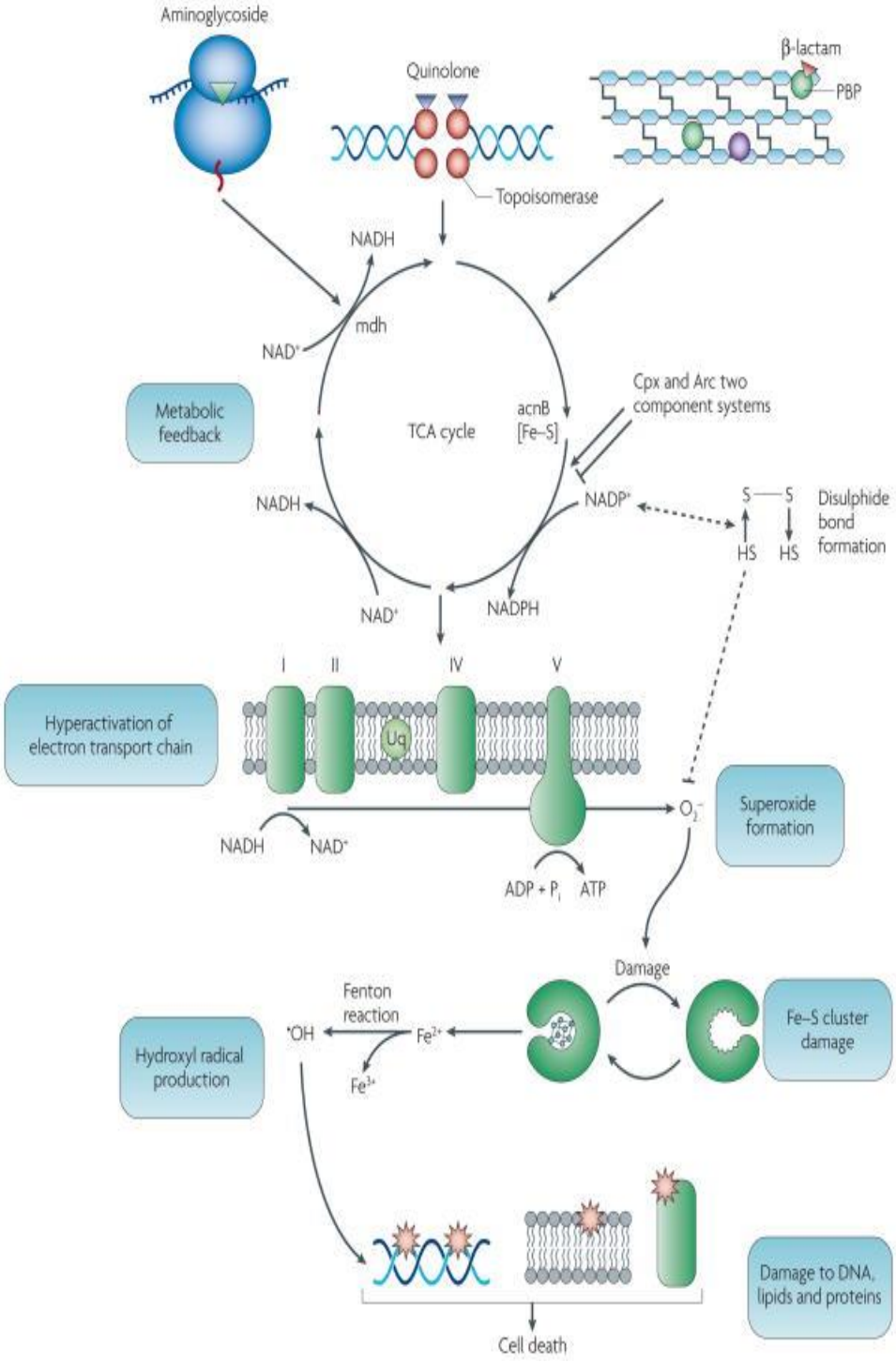


Figure 3: Common mechanism of cell death induced by bactericidal antibiotics (Kohanski et. al.*, 2010).*

Antimetabolites are the molecules that competitively inhibit the use of the metabolite in certain metabolic pathways associated with the survival of bacteria. The antimetabolites, sulfa drugs and trimethoprim (TMP) like drugs compete with p-aminobenzoic acid and also inhibit dihydropteroate synthase and dihydrofolate reductase thus preventing folic acid synthesis. These also inhibit carbonic anhydrases,

which catalyze carbondioxide hydration to bicarbonate and protons (Capasso and Supuran, 2014).

2.2.5 Common mechanism of cell death

Aminoglycosides-ribosome, Quinolones-DNA gyrase, and β-lactams with penicillin-binding proteins are some drug-target interactions which stimulate the oxidation of NADH through the electron transport chain. This leads to reduction of cofactor pool that is required for cellular growth thus increases TCA cycle in increasing pool of NADH and creating vicious cycle of electron transfer. Hyperactivation of the electron transport chain stimulates superoxide formation. Thus formed superoxides damage iron-sulfur clusters, releasing ferrous iron which then undergoes oxidation by the Fenton reaction. The Fenton reaction in turn leads to hydroxyl radical formation which damage DNA, proteins and lipids thus, contributing to cell death. These antibiotics also trigger hydroxyl radical formation and cell death through the Cpx and Arc, the two-component systems (Kohanski *et. al.*, 2010).

2.3 Review of literature related to *Streptomyces* and its potential in antibiotics production

2.3.1 Streptomycetes

Members of the genus *Streptomyces* are the most complex. One unique feature is the production of active volatile metabolite geosmin that gives them distinct earthy odor (Schöller *et. al.*, 2002). They grow as a mycelium of branching hyphal filaments forming septa at regular intervals, creating a chain of uninucleated spores (Ohnishi *et. al.*, 2008).

They produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia (Flärdh and Buttner, 2009). They are chemoorganotrophic gram positive bacteria, resembles filamentous fungi, have genomes with high GC content and found predominantly in soil and decaying vegetation (Madigan *et. al.*, 2012). These make about 40% of soil bacteria (Hasani *et. al.*, 2014).

2.3.2 Antibiotic production from *Streptomyces*

Strikingly, *Streptomyces* is the largest antibiotic-producing genus that have produced approximately two-thirds of all known antibiotics of microbial origin (Lucas *et. al.*, 2013). Besides antibiotics, *Streptomyces* has the ability to produce bioactive secondary metabolites, such as antivirals agent like abikoviromycin against encephalitis virus produced by *S. abikoensis* (Umezawa *et. al.*, 1951), antihypertensives including Phenacein produced by *S. tanashiensis-zaomyceticus* group which is an inhibitor of

angiotensin-converting enzyme (ACE) (Liu *et. al.*, 1984) , antifungals including Amphotericin B isolated from *Streptomyces nodosus* (Caffrey *et. al.*, 2001), immuno-suppressants including Pentalenolactone I and Hygromycin A produced by *Streptomyces filipinensis* and *Streptomyces hygroscopicus,* respectively (Uyeda *et. al.*, 2001) and antitumorals (Ahmad *et. al.*, 2017) making them even more interesting. Almost all of the bioactive compounds produced by *Streptomyces* are initiated during the time coinciding with the aerial hyphal formation from the substrate mycelium (Bérdy, 2012), termed as idiophatic growth.

Figure 4: Key dates of Antibiotics produced by different Streptomyces species (Procópio et. al., 2012).

The growth condition and energy source carbon appear to influence antibiotics production. Galactose, a monosaccharide was found to be the most suitable carbon source for kanamycin production by *Streptomyces kanamyceticus* (Basak and Majumdar, 1973). Furthermore, some researchers have reported that in some of the species glucose tends to decrease antibiotics production by suppression of enzymes involved in antibiotic biosynthesis. However, *Streptomyces anandii* var. *Taifiensis* showed the highest antibiotic level on glucose followed by starch, no considerable antibiotic production on other tested monosaccharides and disaccharides (Lounès *et. al.*, 1996).

Moreover, growth on polysaccharides elevated the antimicrobial production by *Streptomyces noursei* (Jonsbu *et. al.*, 2002). In addition, glucose seems to repress the formation of antibiotics in most of the cases by repressing the antibiotic biosynthetic enzymes (Ruiz *et. al.*, 2010). Thus, choice of energy source appears to be pivotal in antibiotics production.

Thus, identifying proper nutrient sources are critical for antibiotics production. Carbon and nitrogen sources, oxygen, pH, temperature, divalent ions like $Mn2^{+}$, $Cu2^{+}$, $Fe2^{+}$ (Gesheva *et. al.*, 2005) are critical for secondary metabolilte formation by *Streptomyces* and such other factors could affect the production of antibiotics by *Streptomyces.* All these requirements differ as per the species of *Streptomyces* (Shepherd, 2010) so multiple trials should be run altering these factors to increase the antimicrobial productivity from an unknown *Streptomyces* strain.

2.4 Review of literature related to identification of drug targets

Identifying the biological origin and molecular mechanism of a disease and thereafter the potential targets for intervention is the very first step in the drug discovery process. A precise evaluation of mechanism of action (MOA) of bioactive lead molecules against their potential targets is a crucial step during drug development for faster optimization and also for identification of probable off-target side effects thus, helps prevent possible withdrawal of leads in the later process of drug discovery (Schenone *et. al.*, 2013).

Target identification can be attained by various direct biochemical methods, genetic interaction methods or computational inference methods. The direct methods involve the labeling of target proteins or lead molecules of interest, incubation of the two and detection of binding (Lomenick *et. al.*, 2011). In genetic interaction methods, the presumed targets are modified (gene deletions, gene mutations etc.) that could change the sensitivity of lead molecules towards the targets. Computational inferences can help hypothesize a probable MOA by comparison of the lead molecules to the known reference molecules. A chemo-informatics similarity ensemble approach (SEA) and similar approaches can be used to predict the ability of a molecule to bind a target. This is based on the similarity of its chemical groups to ligands of known targets and has been extensively used to find MOA of unknown and/or known drugs (Keiser *et. al.*, 2007; Keiser *et. al.*, 2009; Gregori-Puigjane *et. al.*, 2012; Lim *et. al.*, 2016).

Besides, microscopy based methods could also be used to predict MOA of drugs by characterizing the observable phenotypic changes in the target cells and/or proteins caused by the drugs. In case of anticancer agents, bleb formation can be an indication of disruption of plasma membrane by the drug molecules (Majumdar *et. al.*, 2001). Acyldepsipeptides (ADEPs), which target the core unit of a major bacterial protease ClpP, inhibits bacterial cell division due to uncontrolled proteolysis. Filamentation of *Bacillus*

subtilis when incubated with ADEPs has been reported (Brötz-Oesterhelt *et. al.*, 2005). Also, an unusual altered, elongated *Mycobacteria* was observed by the action of griselimycins produced by *Streptomyces* (Fetz *et. al.*, 2016). This was later on found to be due to inhibition of DNA sliding clamp, DnaN, by genome sequencing techniques (Kling *et. al.,* 2015). However, this method of MOA evaluation is still limited due to inadequate well-characterized reference datasets.

2.4.1 Computational Systems biology approach to drug discovery

Systems biology is the approach where the computational modeling of the complex biological systems is done to understand possible biochemical or phenotypic characteristics. The focuses on understanding a biological system's omics and dynamics would still require a lot of time and resources to generate such database. Even though understanding genes and proteins continue to be of great value but works on entire genes and proteins and subsequent modifications is still a limiting factor. In addition, work on multiple genes or proteins in different metabolic pathways could be limiting. A biological system is not just an assembly of genes and proteins so, manipulating a system requires more than drawing their interconnections. Thus, knowing the overall properties of the system, the patterns involved and how it can be controlled are necessary and computational biology approach for integrating available data could be an alternative for developing system biology in understanding the pathogens.

Ideker *et. al.* (2001) defined Systems Biology as, "The study of biological systems by systematically perturbing them (biologically, genetically, or chemically) for monitoring the gene, protein, and informational pathway responses that on integrating and ultimately, formulating mathematical models could describe the structure of the system and its response to individual perturbations." Thus, a system-level understanding of a biological system can be attained by the knowledge of following properties (Kitano, 2002):

i) System structures: include the network of gene interactions and biochemical pathways

ii) System dynamics: include understanding the system behavior over time under various conditions

iii) Control method: include modulating mechanisms that systematically control the state of the cell, providing potential therapeutic targets in return.

iv) Design method: include devising strategies to modify the biological systems for attaining desired properties based on defined principles and simulations, rather than blind trial-and-error.

Several attempts to create a large-scale, comprehensive database on metabolic pathway, gene-regulatory and biochemical networks are under way. Some examples include KEGG (http://www.genome.jp/), EcoCyc (https://ecocyc.org/), MetaCyc (https://metacyc.org/), BiGG (http://bigg.ucsd.edu/). In addition, protein-protein interaction databases - IID (http://iid.ophid.utoronto.ca/iid/Search_By_Proteins/), MINT (https://mint.bio.uniroma2.it/) and STRING (https://string-db.org/) have been developed to predict possible interacting proteins to the particular protein of interest. Similarly, metabolic network has also been developed. This can help in the dynamic analysis of cellular systems by generating a model initially and subsequently allow applying need base various analytical methods. Different networks and database links are presented in Appendix 8.1.

In particular, constraint-based analysis of metabolic networks have gained a wide popularity for the simulation of various cellular metabolic pathways, of which flux balance analysis (FBA), is among the most widely used constraint-based approach to modulate the properties of metabolic networks (Kauffman *et. al.*, 2003).

2.4.1.1 Reconstruction of metabolic networks and Constraint based modelling

Metabolic reconstruction involves identification, categorization, and inter-connection of the various components of the metabolic network or pathway of a biological system. This integrates genes, proteins, reactions and metabolites to form a network. Such reconstructed models are called the Genome-scale metabolic models (GEMs). GEMs act as a connecting link between genome-derived biochemical information in creating a metabolic network structure and observed metabolic phenotypes (Figure 5). Generally, GEMs describe the entire metabolic reactions (pathways) of an organism at genome-scale using stoichiometric coefficients of the involved metabolites as well as gene-protein-reaction (GPR) associations even if some of the genes and their subsequent transcribed protein functions are unknown by homology modelling through some known similar types of proteins (Bordbar *et. al.,* 2014).

Various genome-scale metabolic models have already been developed for several organisms and constraint based analysis of these reconstructed metabolic networks from FBA in calculating the biomass (Kauffman *et. al.*, 2003) could be one of the tools to use and have proved to be quite useful in various applications including metabolic engineering (Alper *et. al.*, 2005), drug-target identification (Raman *et. al.,* 2005) upon adding different constraints like gene deletion studies (Xu *et. al.,* 2009) in giving new protein targets.

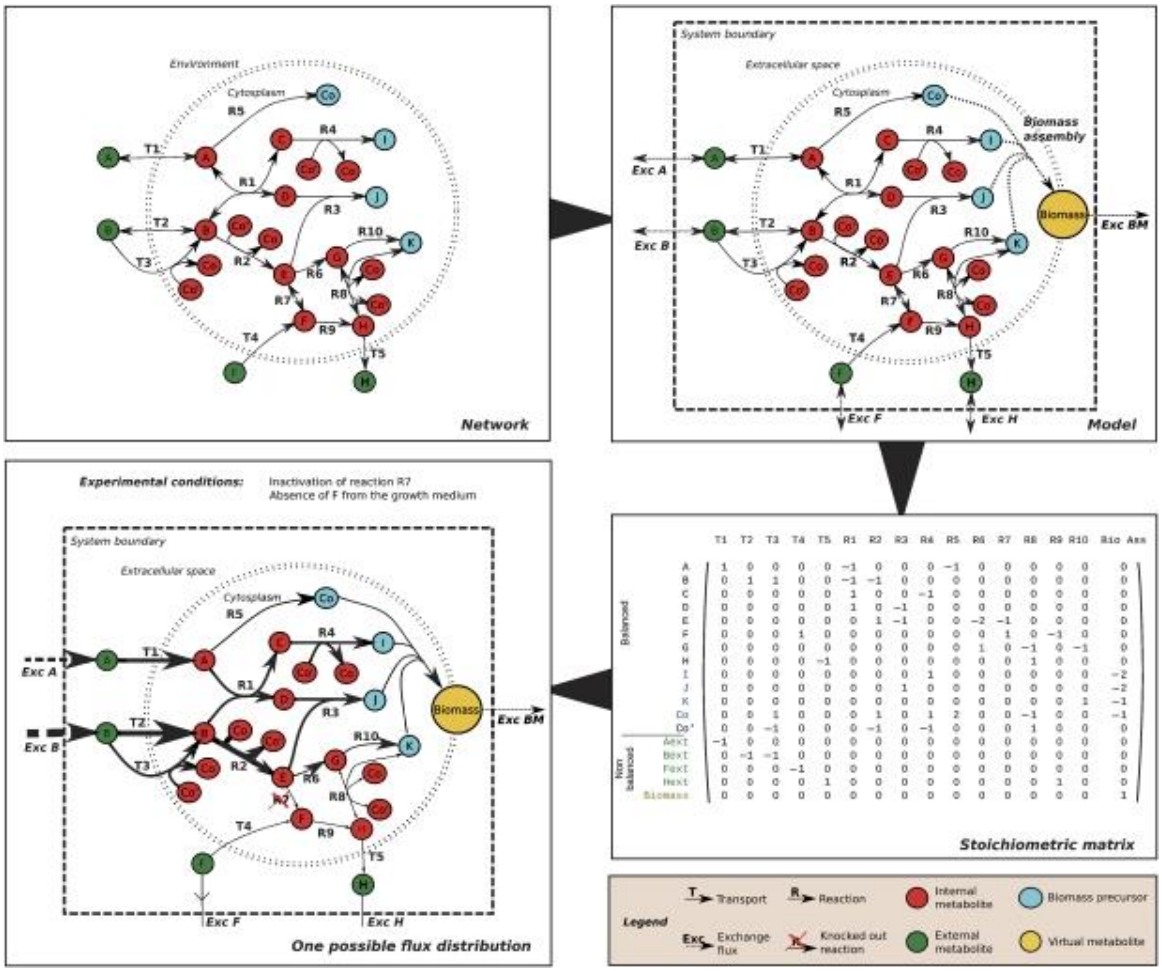


Figure 5: Basic outline of Genome-scale metabolic model reconstruction (Durot et. al., 2009).

Top left – shows a typical example of a metabolic network. Here fate of a particular molecule in the cell can be explained such as transport of sulfur, amino groups, phosphates etc. can be thought of.

Top right – By defining the boundaries of the system, a biomass assembly reaction, and fluxes exchange with the environment, metabolic network is transformed into a metabolic model. Here a precursor molecule, let us say glucose, when supplemented in the growth medium it enters the cell through transporters and is subsequently metabolized into several intermediate precursors such as pentose, erythrose, three carbon and subsequently five carbon metabolites from where RNA and DNA nucleic acids, all the amino acids, lipids and cofactors are made by assimilating nitrogen and sulfur obtained from different sources in increasing biomass (virtual metabolite).

Bottom left – Here altering the amount of particular precursors or ligands, the fate of the biomass (consequent flux distributions) can be displayed proportional to the applied constraints

Bottom right – shows a corresponding stoichiometric matrix used to define all the involved metabolites and each reactions.

Integrating the gene deletion essentiality assay in bioinformatics (Li *et. al.*, 2011; Perumal *et. al.*, 2011) have made possible for the genome-wide annotations of protein sequences for a number of organisms, providing sublime starting points for reconstruction of metabolic networks (Hou *et. al.*, 2016) and would provide the importance of such deletions (Presta *et. al.*, 2017) in identifying lead protein targets.

2.4.1.2 Flux balance analysis (FBA)

FBA is one of the most widely used method of metabolic modelling via constraint-based approach. This uses linear optimization to determine the steady-state reaction flux distribution in a metabolic network by maximizing an objective function, such as ATP production or growth rate. FBA is a mathematical approach for studying biochemical networks, the GEMs in particular, by calculating the flow of metabolites through these metabolic networks. This allows the researchers to predict growth rate of an organism under study and also help increase the production rate of biotechnologically important metabolites (Orth *et. al.*, 2010).

Generally, FBA involves four basic steps:

i) system definition,

ii) obtaining reaction stoichiometrixes,

iii) defining objective function and addition of constraints and

iv) optimization as shown in (Figure 6).

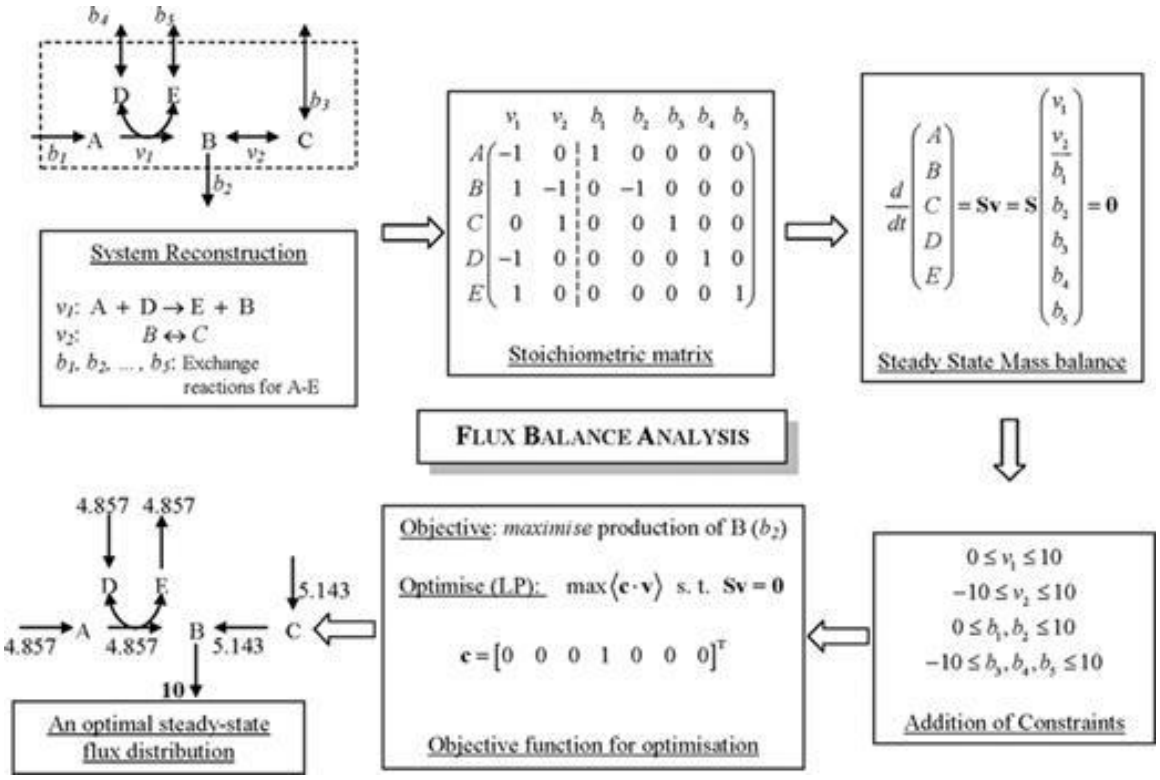


Figure 6: Overview of the steps involved in FBA (Raman and Chandra, 2009).

Metabolic reactions are represented as a stoichiometric matrix (S), of size m*n. Each rows and columns represent the involved metabolites and reactions, respectively. Each of the entries are the stoichiometric coefficients of the involved metabolites in that particular reaction. The negative value refers metabolites consumed while positive coefficient denotes every metabolite that is produced. The metabolites not involved in a particular reaction are given a stoichiometric coefficient value zero. The flux through the reactions is represented by vector v, with length n.

Constraints may be physico-chemical, spatial/topological, condition dependent environmental, or regulatory (Price *et. al.*, 2004). More commonly, additional constraints are imposed on a model by defining the lower and upper bound for the fluxes as follows:

$$0 < v_i < \infty$$

$$-\infty < b_i < \infty$$

which necessitates all internal irreversible reactions to have a positive flux and allows flux exchange in either direction.

The next critical step is to define an objective function for optimization. The linear programming (LP) problem to the search for the optimal flux distribution determination through a metabolic network can be formulated as follows:

Maximize	$Z = c^T v$
Subject to	$S. v = 0$
$\alpha_i \leq v_i \leq \beta_i$	for all reactions i

Here, Z represents the objective function, c^T is transpose of the vector of weights imposed on the fluxes (v). The weights are used to define the properties of the particular solution in demand. S is the stoichiometric matrix and α_i and β_i represents lower and upper bound constraints respectively for each reaction flux v_i.

The solution to this problem yields not only the objective function but also results optimal flux distribution 'v' that allows the highest flux through the chosen objective function, Z (Fu and Panke, 2009).

The steady state growth (Sv = 0) is assumed in FBA where the internal metabolites produced are consumed in a cellular biomass-dependent manner and also mass balance where total biomass of a system remains constant i.e., input of a biomass equals its corresponding output (Orth *et. al.*, 2010). Thus, the variation in biomass yield upon constraint provided would render whether that metabolite is essential or not, hence, the gene is essential.

If a gene is deleted or if the condition is created when a particular metabolite is provided as zero then the biomass yield would say whether that metabolite is essential or not and the gene responsible for that particular metabolism is essential. For example if glucose value is zero and xylose value is in positive and net biomass yield or cell biomass is in positive then in this condition xylose transporter and enzymes involved in xylose metabolism to yield ribulose-5-phosphate is critical. Among them also which enzymes is the rate limiting can be calculated by deletion of respective genes and biomass calculation. Various FBA tools are mentioned in Appendix 8.2.

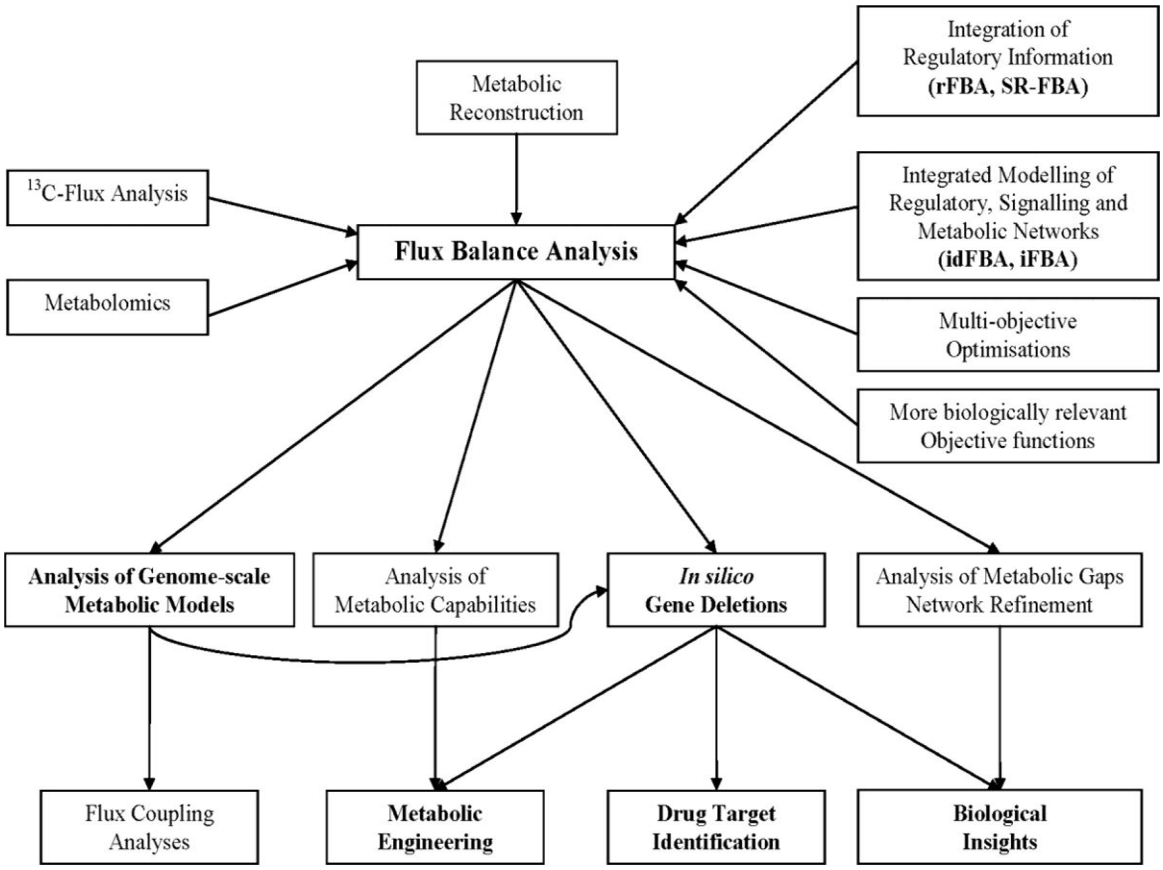


Figure 7: Extensions and applications of FBA (Raman and Chandra, 2009).

2.5 Review of literature related to Vitamin B12 biosynthesis pathway as a probable drug target

Vitamin B12 (cobalamin) is a *cobal*t-containing modified tetrapyrrole, produced mainly by prokaryotes (Martens *et. al.*, 2002) and some plants, that is an essential cofactor for several important enzymes catalyzing various transmethylation and rearrangement reactions. Being the most complex of all the other vitamins, it is very expensive in terms of energy to synthesize since there are more than 30 biochemical steps required (Scott and Roessner, 2002). Thus, vitamin B12 can be considered highly valuable for the

bacterial survival and as a probable therapeutic target, a potential solution to the increasing resistance problems.

2.5.1 Vitamin B12 biosynthesis

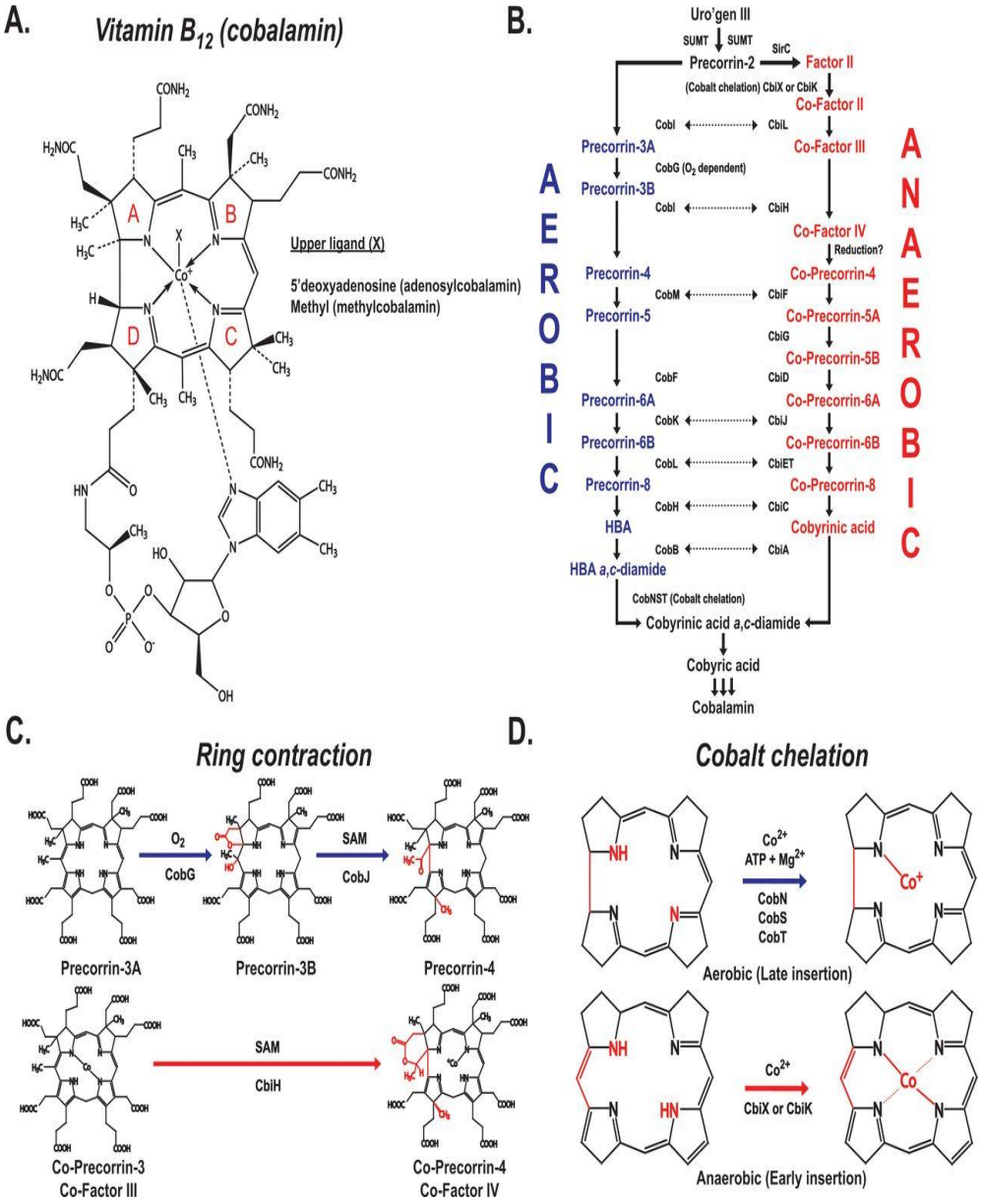


Figure 8: Summary of the aerobic and anaerobic corrin ring contraction and cobalt chelation in Vitamin B12 *biosynthesis pathways (Moore and Warren, 2012).*

(A) Structure of vitamin B12 (B) Aerobic and anaerobic pathways. Similarities shared between Cob and Cbi enzymes are indicated by broken arrows. (C) Ring contraction. (D) Simplified diagram of *coba*lt chelation at tetrapyrrole ring.

The overall transformation of uroporphyrinogen III into tetrapyrrole containing vitamin B12 requires a number of peripheral C-methylations, ring contraction with the loss of the C-20 *meso*-position, *coba*lt chelation, amidation of the majority of the carboxylic acid side chains, decarboxylation of the acetic acid side chain on ring C, aminopropanol attachment, adenosylation and attachment of the lower base in the form of α-ribazol with the central corrin ring (Warren *et. al.*, 2002).

A total of eight S-adenosyl-L-methionine (SAM) mediated methylation (primary focus of our research) has been reported to transfer the methyl groups to the uroporphyrinogen framework, although only seven are observed in the final product.

Some of the Vitamin B12 biosynthetic genes and SAM biosynthetic genes exhibit riboswitch structure at 5'-UTR region of mRNA modulating peptide biosynthesis even mRNA is transcribed.

2.6 Review of literature related to Riboswitches

Riboswitches are the elements commonly found in the 5'-untranslated region (UTR) of mRNAs that bind small molecule ligands and regulate the mRNA activities *(*Mironov *et. al.*, 2002).

2.6.1 Structure of Riboswitch

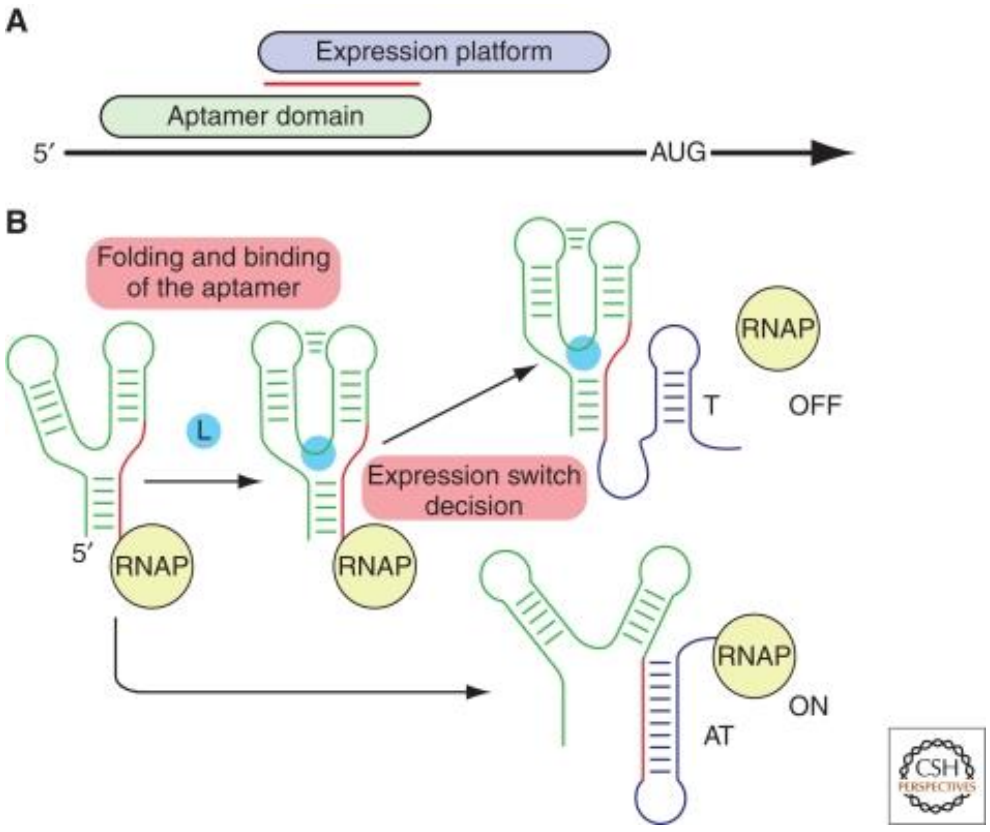


Figure 9: An overview of organization and mechanism of the typical riboswitch (Garst et. al., 2011).

A typical riboswitch consists two distinct functional domains – aptamer domain and expression platform. Aptamers are short single stranded nucleic acids which recognize the effector molecule (ligands) and function as a ligand binding pocket by adopting a compact three-dimensional fold with very high binding affinity and specificity (Weigand and Suess, 2009). The on and off states of the mRNA are represented by either of the two mutually exclusive structures formed in the expression platform which is regulated by a region of overlap between these two domains (Figure 9; Garst *et. al., 2011*), referred as switching sequence (Hennelly *et. al.*, 2013). A second domain, the expression platform, undergoes structural changes in response to the changes in aptamer regulating gene expression (Figure 10; Price *et. al.,* 2014).

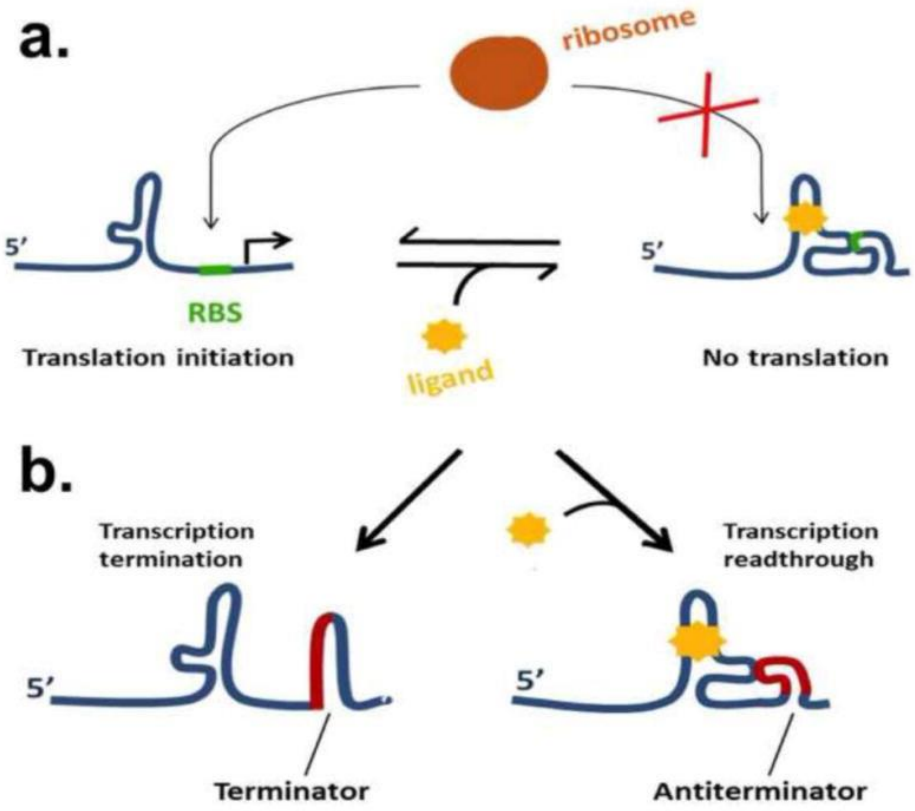


Figure 10: Schematics of riboswitch-mediated gene regulatory circuits (Price et. al., 2014).

Various riboswitches have been experimentally validated till date, including

- Cobalamin riboswitch – binds either adenosyl cobalamin or aquocobalamin and regulates cobalamin biosynthesis and transport of mostly cobalamin (Nahvi *et. al.*, 2004).

- SAH riboswitch – binds S-adenosyl homocysteine and regulate genes essential for recycling expended SAM coenzymes (Wang *et. al.*, 2008).

- SAM riboswitch – binds S-adenosyl methionine (SAM) and regulates methionine and SAM biosynthesis and transport (Batey, 2011).

- Lysine riboswitch – binds lysine and regulates its biosynthesis, catabolism and transport (Blouin *et. al.*, 2011).

- Cyclic di-GMP riboswitch – binds c-di-GMP and regulates many genes related to c-di-GMP metabolism as well as genes involved in bacterial motility, virulence and biofilm formation (Smith and Strobel, 2011).

- Tetrahydrofolate riboswitch – binds tetrahydrofolate and controls transport and biosynthesis of folate and its derivatives in Firmicutes (Trausch *et. al.*, 2011).

- Fluoride riboswitch- binds fluoride ions and function in surviving high levels of fluoride (Ren *et. al.*, 2012).

- glmS ribozyme- riboswitch – binds glucosamine-6-phosphate (GlcN6P) and permits feedback regulation of GlcN6P levels (McCown *et. al.*, 2012).

- Purine riboswitches – bind purines including guanine, adenine and 2' – deoxyguanosine and regulate purine metabolism and transport (Batey, 2012).

- TPP riboswtich – binds thiamine pyrophosphate (TPP) regulating thiamine biosynthesis and transport (Haller *et. al.*, 2013) and is the only riboswitch so far in Eukaryotes (Bocobza and Aharoni, 2008).

- PreQ1 riboswitch – binds pre-quenosine and help regulate the biosynthesis and transport of PreQ1, in a number of Firmicutes, Proteobacteria and Fusobacteria (Eichhorn *et. al.*, 2014).

- Cyclic di-AMP riboswitch (also called ydaO/yuaA) – binds second messenger molecule cyclic di-AMP involved in signaling DNA damage and cell wall stress (Gao and Serganov, 2014).

- Glycine riboswitch – binds glycine and regulates glycine metabolism genes (Ruff and Strobel, 2014).

- Cyclic AMP-GMP riboswitch – binds secondary messenger molecule cyclic AMP-GMP and control many genes involved in utilization of extracellular iron (III) oxide as an electron sink by various *Geobacter*species (Nelson *et. al.*, 2015).

- FMN riboswitch- binds flavin mononucleotide (FMN) and regulates riboflavin biosynthesis and transport (Pedrolli *et. al.*, 2015).

- Glutamine riboswitch - binds glutamine and regulates genes involved in nitrogen metabolism (Ren *et. al.*, 2015).

a) Illustration of a typical translational riboswitch in which ligand binding prevents translation initiation by blocking RBS.
b) Illustration of a transcriptional riboswitch in which ligand binding to aptamer domain causes the effector domain (red) to adopt an anti-terminator conformation, thereby preventing premature transcription termination.

2.6.2 SAM Riboswitches

SAM is a key methyl donor synthesized from methionine and ATP, involved in the methylation of various macromolecules including DNA, RNA, proteins, phospholipids, hormones and neurotransmitters (Grillo and Colombatto, 2008). SAH is the by-product of SAM mediated methylation and both of their riboswitches extensively regulate sulfur metabolism in many bacteria (Wang and Breaker, 2008).

SAM-I riboswitch facilitates the formation of an intrinsic terminator stem upon SAM binding, resulting the termination of transcription (Winkler *et. al.*, 2003). A variation of the SAM-I class, called SAM-IV was later discovered with similar binding pocket and phylogenetic distribution as SAM-I presently, primarily in the order Actinomycetales but differs in the scaffolding beneath the binding nucleotides (Weinberg *et. al.*, 2008).

SAM-II riboswitches found predominantly in α-proteobacteria (Corbino *et. al.*, 2005), are typically short sequences which form an H-type pseudoknot upon SAM binding with entirely distinct sequence and structural features from those of SAM-I and SAM-IV. The pseudoknot ends 2 nt upstream of the Shine-Dalgarno (SD) sequence but is sufficient to occlude the ribosome binding in “off ” state (Gilbert *et. al.*, 2008).

SAM-III or S_{MK}-box is also a translational riboswitch, narrowly distributed mainly in the order Lactobacillales (Fuchs *et. al.*, 2006). Like SAM-II riboswitch, it regulates gene expression by sequester ribosome binding site (RBS) or SD sequence. Upon SAM binding, the SD base pairs with an anti-SD sequence to form aptamer, thus preventing the binding of ribosome in the RBS. SAM-III aptamers yield a tertiary structure and binding pocket which are distinct compared to other SAM riboswitches (Lu *et. al.*, 2008).

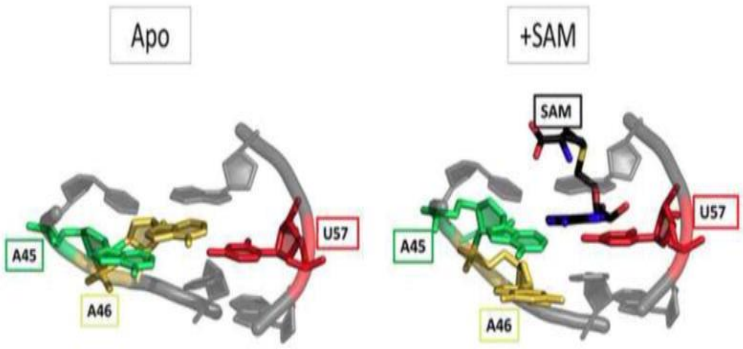


Figure 11: SAM-I binding site with and without SAM (Stoddard et. al., 2010).

When SAM (black) binds, it forms a base triple with A45 and U57 (green and red). In the apo state, this interaction is blocked by A46 (yellow).

2.7 Review of literature related to Virtual Screening

2.7.1 Virtual Screening and its role in drug discovery

A typical drug discovery cycle, from lead identification to clinical trials is estimated to take around 14 years (Myers and Baker, 2001) and costs approximately 2.6 billion US dollars as reported by Tufts Center for the Study of Drug Development (CSDD) in 2014 (https://www.scientificamerican.com/article/cost-to-develop-new-pharmaceutical-drug-now-exceeds-2-5b/) thereby demanding alternative drug discovery approaches. This is where Virtual Screening comes into play to limit the excessive time and resources consumed during drug discovery.

Virtual Screening (VS) is a computational technique used in drug discovery applicable in filtering and assessing large libraries of chemical structures and/or combinatorial libraries. This acts as an important tool to guide the search for drug-like candidates. Simply, VS shortens the number of compounds and thus the time taken for evaluation by High Throughput Screening (HTS) in a significantly less expensive cost. VS has become a cornerstone in modern drug discovery also because of the increasing accuracy rates of these methods with the advent of newer technologies (Thomsen and Christensen, 2006; Trott and Olson, 2010; Gu *et. al.*, 2015)

2.7.1.1 VS types

There are two broad categories of Virtual Screening techniques: ligand based (LBVS) and structure based (often referred as docking) (SBVS). In the present study both kinds of VS were done to specifically look for a group of compounds that have been reported to exhibit kinase inhibition and taking the adenosine moiety of both the target metabolite SAM and the ATP that is required for kinases in screening for potential molecules that could be better inhibitors of some of the enzymes and riboswitch where SAM is bound at the active sites with the higher binding affinity as competitive inhibitors.

i) Ligand-Based Virtual Screening (LBVS) :

Ligand-based methods screen molecules with common physical and chemical properties to the known binding compounds or inhibitors of a pharmacological target based upon the assumption that similar compounds could exhibit similar effects (Stahura and Bajorath, 2005). These methods are usually employed when the high-resolution structure of the therapeutic targets are not available. However, known inhibitors (at least one) to the biological targets are strictly required for ligand-based approaches to work. Despite various limitations such as lack of definitive molecular descriptors for screening, limited diversity of the normally encountered hits and prevalence of

structurally very unlike compounds with alike behaviors when bound to receptors, moreover, drug design against human epidermal growth factor receptor 2, HER2, a commonly over-expressed tyrosine kinase receptor found in various carcinomas (Huang *et. al.*, 2010) and other several successful applications in aiding drug discovery have been reported (Geppert *et. al.*, 2010; Mishra *et. al.*, 2015).

ii) Structure-Based Virtual Screening (SBVS):

Structure-based methods have proven to be more effective than traditional methods of drug discovery since these are focused on understanding the molecular basis of a disease and function on the 3D structures of the biological targets (Lionta *et. al.*, 2014). These methods involve the interaction between the ligands and a target (receptors or enzymes or proteins) with a goal to screen out ligands with stronger binding affinity to the targets.

The target protein 3D structure is a must for SBVS, which can be derived from experimental data (X-ray, NMR, or neutron scattering spectroscopy), Molecular Dynamics (MD) simulations, or homology modeling (the structure could be derived through computational modeling by entering the amino acids sequences derived from genomic information).

Various online tools have been developed to predict the tertiary structure of proteins with only the amino acids sequence and an experimental 3-D structure of a related homologous protein (template) including RaptorX (Källberg *et. al.*, 2014), Phyre2 (Kelly *et. al.*, 2015) and so on. RaptorX uses a novel nonlinear context-specific alignment potential and probabilistic consistency algorithm while Phyre2 uses advanced remote homology detection methods to construct 3D models, predict the ligand-binding sites, and also analyze the variations in amino acids in a protein sequence.

2.7.1.2 Steps for a Structure Based Virtual Screening (SBVS)

The typical stages followed in a normal VS process include defining the drug target, preparation of compound library, choice of appropriate filters, docking protocol and post filters to limit the time required to screen and increase the accuracy of the results.

2.7.1.2.1 Defining Drug Target

Accurate description of the target is the first and foremost thing to be considered for a successful SBVS. The classical approach of clearing out the water molecules from the binding pocket can affect the docking results since water molecules have been found to play an important role in ligand-receptor binding in many cases (Günther *et. al.*, 2003). Thus, better parameters have to be devised.

The availability of quality 3D structures of the target also determines the success of SBVS. Usually X-ray crystallographic or NMR structures of target proteins are chosen for

SBVS keeping in mind the appropriate resolution of the diffraction data. Though, a riskier approach, homology models have also been used for successful drug discovery (Evers and Klabunde, 2005). Various aspects regarding targets need to be contemplated before proceeding to further steps that include target selection, its druggability, structure, molecular flexibility, protonation state, and interacting water molecules (Cerqueira *et. al.*, 2009).

Molecular flexibility of protein targets can complicate virtual screening results since protein targets have internal degrees of freedom so can adopt to various conformational states. So flexible molecular docking methodologies though expensive, have been developed and are still under progress (Meng *et. al.*, 2011). Generally, the function of the target, the metabolic pathways regulated by the target, and its essentiality are considered during target selection.

A good drug target must be druggable i.e. amenable to small molecules intervention. Though still scarce, several features to differentiate druggable and non-druggable targets have been suggested examining features like shape, size, polarity and geometry of active sites of the targets (Hajduk *et. al.*, 2005; Yuan *et. al.*, 2013; Kandoi *et. al.*, 2015).

2.7.1.2.2 Compound Library selection

The more the structural/chemical diversity of a database, the higher are the chances of finding useful leads leading to drug discovery. A large variety of compound databases are available in public among which the commonly used ones are as below.

National Cancer Institute, NCI database (350,000 compounds) (Milne *et. al.*, 1994), BIOVIA ACD (over 10 million unique chemicals, including 3D models) and ZINC (over 230 million ready-to-dock compounds) (Sterling and Irwin, 2015).

2.7.1.2.3 Filters

A large dataset of compounds would take an extremely longer time for molecular docking against a particular drug target and also could result in hits that would fail in the later stages of drug development. So, appropriate filters can be used so as to remove the undesirable compounds from the database under study, thus ensuring a more cost and time-effective approach. The pharmacokinetic and Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of a compound would determine the fate of the compounds available in the database under study.

A pharmacological compound has to penetrate through various physiological barriers, such as gastrointestinal barrier, blood-brain barrier (BBB) and microcirculatory barrier in order to reach the blood circulation. Subsequently, it is transported to its effector site for further distribution into tissues and organs, where it is metabolized by the action of certain enzymes, and finally the excess and/or undigested remains is removed out of the

body via excretion. Some compounds may cause adverse reactions or toxicity (Tox) in humans so these are best excluded in the earlier stages of drug development (Gardiner, 2006).

The membrane permeability of a compound is determined by various factors including its size, aqueous solubility, ionizability (pKa) and lipophilicity (logP). Also, polar surface area (PSA) is inversely proportional to the lipid penetration ability (Palm *et. al.*, 1997). Compounds completely absorbed by humans have PSA values of ≤ 60 $Å^2$ (Veber *et. al.*, 2002).

Several rules have been built to ensure drug-likeness properties of a compound, the most popular being Lipinski's rule of five (Lipinski, 2004) which states that an orally active drug has no more than one violation of the mentioned criteria:

Molecular weight < 500 daltons

Lipophilicity (logP) < 5

Hydrogen bond donors < 5

Hydrogen bond acceptors < 10

Apart from filtering for lead-like properties, known toxicophores and metabolically unstable compounds should also be excluded (Blagg, 2006). Moreover, several novel filters for quality enhancement of the databases have been developed recently. The ALARM-NMR filters (Metz *et. al.*, 2007) and the Pan Assay Interference Compounds (PAINS) (Baell and Holloway, 2010) identify chemically reactive and assay-interfering compounds.

Thus, pharmacological and ADMET properties should be considered in the early stages of drug development process for efficient filtering of compound database. Tools such as CLEVER (Chemical Library Editing, Visualizing and Enumerating Resource) supports chemical library creation and manipulation, combinatorial chemical library enumeration using user-specified chemical components, chemical format conversion and visualization, as well as chemical compounds analysis and filtration with respect to drug-likeness, lead-likeness and fragment-likeness based on the physicochemical properties computed from the derived molecules (Song *et. al.*, 2009).

Finally considering the "Jorgensen Rule-of-Three" is another common rule for predicting lead like properties which states that the aqueous solubility measured as logS should be greater than -5.7, the apparent Caco-2 cell permeability should be faster than 22 nm/s, and the number of primary metabolites should be less than 7(Di and Kerns, 2016) could give potentially effective molecule that could enhance the success rate during the drug development in future processes.

2.7.1.2.4 The docking protocol

Molecular docking refers to the ligand-protein docking done in order to predict the predominant binding mode(s) of a ligand with a target protein and several different docking programs have been developed, including ICM (Abagyan *et. al.*, 1994), FlexX (Rarey *et. al.*, 1996), GOLD (Jones *et. al.*, 1997), DOCK (Ewing *et. al.*, 2001), Glide (Friesner *et. al.*, 2004) that require protein of known 3D structure (Morris and Lim-Wilby, 2008).

Rigid docking methods are comparatively faster than flexible docking methods. However, the neglect of ligand and/or target flexibility may result in missed hits due to an inadequate representation of the ligand and/or target conformational space and AutoDock (Morris *et. al.*, 2009) appears to be more flexible in predicting the binding affinity.

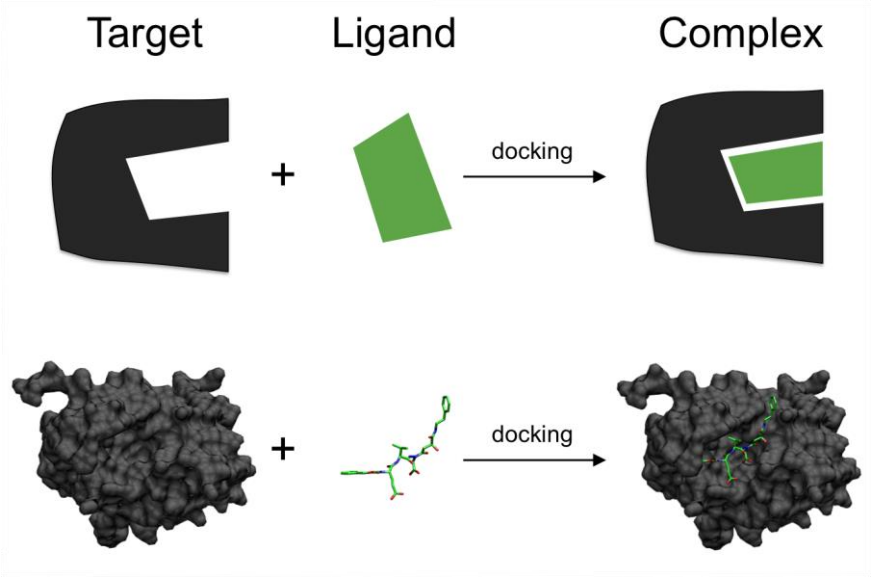


Figure 12: Schematic illustration of molecular docking (protein-ligand docking)

2.7.1.2.5 Post Processing (Improving selection after docking)

After completion of molecular docking, post filters should be applied before selection of compounds for further test phases so as to omit the probable false positive results from docking. The use of simplified scoring functions and sometimes the inadequate sampling of the conformational space for the ligands may lead to unrealistic poses, intra-ligand steric clashes, twisted amides, E/Z esters, imperfect hydrogen-bonding network, and poses based on shape complementarity resulting an unreasonably high scores need to be discarded. Progress have been made in increasing the efficiency and quality of compound selection (Waszkowycz, 2008). Other common post filter criteria include diversity of ligands and practical issues such as price, availability, and quality of the compounds.

Various current improvements in SBVS efficiency have been observed by processes like Ensemble Docking (ED) and Consensus Induced-Fit Docking (cFID). Usually, Crystal structures used in these docking strategies provide a single conformation of the target proteins and are highly influenced by ligand binding, resulting conformational changes in both protein and ligand. Thus, crystal structures despite of being a preferred starting point for SBVS, they could provide misleading information in some cases. This can be solved by alternative docking approaches - ED, docking of a ligand database against multiple rigid receptor conformations (Korb *et. al.*, 2012) along with cFID, that allows the protein binding site to adapt to multiple ligands during SBVS (Kalid *et. al.*, 2012)

3. MATERIALS AND METHODOLOGY

3.1 Screening of *Streptomyces* for Antimicrobials production using Traditional methods

The geographical diversity and ecologically rich niche in Nepal makes it a favorable habitat for many different organisms. Since Nepal carries a huge research potential for finding novel *Streptomyces* and due to lack of adequate research in this sector in Nepal, traditional methods of antimicrobials production were first applied hoping to find novel antimicrobials producers against the growing resistance among the pathogens.

3.1.1 Selection of Soil samples

Different soil samples collected from different parts of Nepal (including Terai, Mountain and Himalayas) and stored in Central Department of Biotechnology, Tribhuvan University were taken for isolation of putative *Streptomyces sps.* with probable potential to produce antimicrobial agents. Soil sample collection was expanded to Panchase area near Pokhara where rare tree ferns have been found based on previous studies and it was presumed novel *Streptomyces* could be present in that habitat.

3.1.2 Primary culture and Pure culture isolation

International *Streptomyces* Project (ISP) media, ISP2 and ISP4 (Shirling and Gottlieb, 1966) supplemented with Cycloheximide, an antifungal agent were used for initial screening of Streptomycetes from soil samples. 10^{-4} dilutions of soil samples in sterile water were plated in sterile ISP2 and ISP4 growth media 100μl each, by spread plating method and incubated at 28°C for atmost 15 days. The growth of the colonies was checked in the plates at regular intervals of 2-3 days. Pure cultures were isolated in ISP2 plates by streaking from the primary plates. Only those colonies which exhibited dry, powdery, non-mucoid or color forming traits were taken for pure culture isolation.

3.1.3 Production of Bioactive metabolites

Putative *Streptomyces* were grown on ISP2 broth for 7-10 days at 30°C and 150 rpm for secondary metabolites production. All the cultures were subjected to secondary metabolites extraction using ethyl acetate as a solvent. Equal volume (1:1) of culture broth and ethyl acetate were agitated for about 30 mins, separated using separating funnel and the metabolites were concentrated using Rotavapour, and cryopreserved as methanol stocks.

3.1.4 Antimicrobial Susceptibility Testing (AST)

3.1.4.1 Selection of test organisms

The American type culture collections of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and carbapenem resistant *P. aeruginosa* PA76 collected by Samikshya Kafle from Teku hospital were selected for antimicrobial susceptibility testing.

3.1.4.2 Standardization of Inoculum

EUCAST guidelines (Matuschek *et. al.*, 2014) were considered for antimicrobial susceptibility testing. The test organisms were grown in non-selective media, Luria-Bertani (LB) broth overnight (16-24 hrs of incubation). The inoculums of test organisms were maintained to the density of a McFarland 0.5 standard, approximately corresponding to $1\text{-}2*10^8$ CFU/ml before the susceptibility tests to ensure the consistency of all the tests performed.

3.1.5.3 Resazurin antimicrobial tests

The protocol mentioned in Resazurin cell viability Assay kit (www.biotium.com) was taken as a reference and modified. The test organism was grown overnight in a non-selective media (LB) and McFarland 0.5 standard was maintained the other day, by necessary dilutions using the LB broth media. In a 96-well microtiter plates, 83µl of culture along with 15µl extract and 2µl of 1% resazurin solution was kept in each well. The final volume was made 100 µl in each wells and final resazurin concentration as 0.02%. All the columns in the microtiter plate had one well without the extracts, included 83µl culture broth, 2µl resazurin and 15µl LB broth. This was used as a negative control for Antimicrobial assay. The extracts showing positive AST results were selected for further tests.

3.1.5.4 Inoculation of agar plates, preparation and application of antimicrobial disks

100µl culture broth maintained at McFarland 0.5 standard was pipetted in MHA plates and spread throughout the agar plate with the help of a cotton swab.

0.5 cm sterile filter paper discs were added with 15µl extracts each, and used as antibiotic disks. The disks were applied firmly on the agar surface within 15 mins of the inoculation of the plates with the test organisms. Each MHA plates were applied with 4 of the prepared antibiotic disks and one negative control, 15µl methanol at the center of the plate. Within another 15 mins of application of antimicrobial disks, the assay plates were inverted and incubated at 37°C for 16-24 hrs based on the types of test organisms (short incubation time for those with lesser generation time).

3.1.5.5 Measurement of Inhibition zone and Result interpretation

After the incubation period, the inhibition zone diameters were measured with a ruler to the nearest millimeter. Zone diameters were interpreted and categorized as susceptible, intermediate or resistant according to the EUCAST clinical breakpoint tables (EUCAST, 2016).

3.2 Search for novel drug targets and Development of leads

3.2.1 Selection of MDR strains and obtaining their genomic sequences

The highly prioritized pathogens that have been published by WHO 2017 as Critical and High priority (Lawe-Davies and Bennett, 2017) against whom new antimicrobials are sought were taken as the reference organisms. The whole genome sequences of these organisms or their closely related ones, which are published in NCBI were taken for whole genome alignment.

The genomic sequences of the 9 selected pathogens on the basis of WHO priority list, were downloaded from NCBI FTP site in the annotated gbk format.

ftp://ftp.ncbi.nlm.nih.gov/genomes/archive/old_genbank/Bacteria/

3.2.2 Multiple Sequence Alignment:

Multiple Sequence Alignment was performed using the progressive Mauve algorithm in MAUVE, a multiple sequence alignment software. The genomic regions common to all the aligned sequences were searched for, in MAUVE via visual observation of locally collinear blocks (LCBs) denoted by certain color codes. LCBs represent homologous regions of sequence shared by two or more of the genomes under study without rearrangement (Darling *et. al.*, 2004).

3.2.3 Alignment of Amino acid sequences of the lead proteins:

Clustal Omega was used to align the amino acid sequences of the lead proteins S-adenosyl methionine synthase (*metK*) and Uroporphyrinogen-III methyl transferase (*cobA*) for all 9 selected pathogens.

3.2.4 Gene essentiality analysis:

The common genes obtained from MAUVE alignment, were looked for their essentiality. The available genome scale metabolic models of four out of above 9 organisms, were subjected to gene deletion studies by converting their respective models into a stoichiometric model and performing flux balance analysis (Varma and Palsson, 1994).

The reconstructed metabolic network of *Pseudomonas aeruginosa* was obtained which consists 1129 genes, 1495 reactions and 1286 metabolites (Bartell *et. al.*, 2017), that of

Salmonella typhimurium called STM_v1.0 contained 1270 genes, 2,201 network reactions, 738 transport reactions, 1,463 Biochemical reactions and 1,119 metabolites (Thiele *et. al.*, 2011), that of *Staphylococcus aureus*, iSB619 contains 619 genes that catalyze 640 metabolic reactions (Becker and Palsson, 2005), and that of *Helicobacter pylori* iIT341 accounts for 341 metabolic genes, 476 intracellular reactions, 78 exchange reactions, and 485 metabolites.

For each gene, the associated reaction(s) were disabled ($v_{min,\ i} = v_{max,\ i} = 0$ mmol.gDW^{-1}.hr^{-1}) and the ability of the model to produce biomass was accessed, i.e., the biomass reaction was chosen as the objective function and maximized (Thiele *et. al.*, 2011). The associated genes of the reactions for which the biomass production is zero, were marked as essential genes.

All simulations were performed using the COBRA Toolbox v2.0 (Schellenberger *et. al.*, 2011) using Matlab (Mathworks, Inc) as the programming environment, and GNU Linear Programming Kit (GLPK) as the linear programming solver.

3.2.5 Molecular docking simulation:

3.2.5.1 Obtaining the dockable crystal structures of the target proteins

The lead proteins were selected from one of the targeted pathogens since the active binding sites of these proteins were found to be similar in all these aligned pathogens. The X-ray diffraction structures of S-adenosyl methionine synthase from *Neisseria gonorrhoeae* with bound S-adenosyl methionine, AMP, Pyrophosphate, Phosphate and Magnesium (PDB id: 5T8S) (Dranow *et. al.*) ; Uroporphyrin-III C-methyl transferase from *Thermus thermophilus* complexed with S-adenyl homocysteine (not available for other organisms) (PDB id: 1V9A) (Rehse *et. al.*, 2005) and that of SAM-I riboswitch from *Bacillus subtilis* with bound SAM and magnesium (PDB id: 4KQY) were obtained from Protein Data Bank (rcsb.docking.org) in PDB format.

3.2.5.2 Preparation of ligand database

In the present work, both ligand based and structure based virtual screening was performed. LBVS was done because similar compounds exhibit similar physico-chemical and biological properties so a broad chemical database with structural diversity would offer an ideal solution for effective lead discovery. In one of the works (Wu *et. al.*, 2005) indirubins have been found to be kinase inhibitors. In earlier works, assuming kinase inhibitors could be a potential compound that could compete with SAM due to adenosine moiety, NCI diversity database II was screened and 4-methyl-N-(3-phenylphenyl)piperazin-4-ium-1-carboxamide, an indole derivative had binding energy higher than SAM in MetK *protein*. This showed that the indole containing compounds could be potential leads against pathogens. In this study, a ligand database containing

715 molecules was prepared that included Indole and Indirubin derivatives from ZINC database (Irwin and Shoichet, 2005).

3.2.5.3 Protein and ligand preparation

SBVS was perfomed based on the common gene in all nine pathogens, *metK*, and the metabolite that it produces, SAM which is further utilized in methylation reactions. Prior to molecular docking, the proteins and ligands need to be prepared for efficient and more accurate docking results. Protein preparation is done by adding hydrogen atoms, merging non-polar bonds, adding Gasteiger charges in mgltools (http://mgltools.scripps.edu/). Similarly, ligand preparation was done in Openbabel GUI (O'Boyle *et. al.*, 2011) available in PyRx interface by energy minimization and converted to pdbqt file format, a useable file format for docking afterwards.

3.2.5.4 Setting reference values for docking

The native ligands were removed from each of the target proteins in Discovery Studio Visualizer 2017 and docked back in their binding sites, a process called re-docking. The highest binding energy thus calculated was taken as a reference value for identifying potential leads from the ligand database, docked against the respective binding pockets of the target proteins.

3.2.5.5 Structure based Virtual Screening

Virtual Screening was carried out using AutoDock Vina in a virtual screening software, PyRx (Dallakyan and Olson, 2015) against the target proteins with the selected ligand database. The conformation with the lowest docked energy was chosen after the docking interactions and visualized and analyzed using Discovery Studio.

3.2.5.6 ADME/Tox Screening

Drug discovery and development are very expensive processes, many promising lead compounds fail to reach the clinical stage of drug development mainly due to poor pharmacokinetic properties as well as toxicity issues. The toxic profiles and druglikeness based on the binding energies, were predicted using OSIRIS program (Nisha *et. al.*, 2016). OSIRIS calculate various drug relevant properties like molecular weight, cLogP, cLogS, Druglikeness, and toxicities like mutagenicity, tumorigenicity, reproductive effects and irritant effects in the lead molecules on the basis of functional groups present in their structures (Sander *et. al.*, 2015).

3.3 Primer Designing

3.3.1 Primer Design

A specific primer for Uroporphyrinogen-III methyl transferase *cobA* gene promoter from *Pseudomonas aeruginosa* was designed manually using various web-based tools namely,

Oligo Calc (Kibbe, 2007), OligoAnalyzer 3.1 (Owczarzy *et. al.*, 2008) and The mfold Web Server (Zuker, 2003) considering the guidelines for efficient primer design regarding length, GC content, melting temperature (Tm), 3' end complementarity, homo and heterodimers formation etc.

The length of *cobA* promoter was not experimentally validated till date. so, the forward primers were designed 100 bp, 200 bp and 500 bp upstream of the Transcription Start Site (TSS) (Appendix 8.3) to know the presence of potential SAM riboswitch in *cobA* and also to check whether the lead candidates developed, affects *cobA* promoter or not.

4. RESULTS AND DISCUSSION

4.1 Isolation and characterization of putative *Streptomyces*

As recommended by International *Streptomyces* Project (ISP), ISP2 and ISP4 growth media (Shirling and Gottlieb, 1966) supplemented with Cycloheximide, an antifungal agent (Gavrish *et. al.*, 2008) were used for isolation of putative *Streptomycetes* sps. from different soil samples. Since soil is a reservoir of a huge number of microorganisms and *Streptomycetes* is a slow grower that generally takes around 7-15 days in culture media (Kim, 2013; Zothanpuia *et. al.*, 2017), the soil sample that was re-suspended in sterile distilled water was further diluted to 10^{-4} to reduce the crowding of organisms.

The ISP4 media that contained soluble starch as sole reduced carbon source allowed growth of the bacteria that can use this as sole carbon source, mostly *Actinomycetes* (Zainal *et. al.*, 2016), and eliminated growth of other bacteria that are dependent on easily metabolizable reduced sugars for their growth (Wawrik *et. al.*, 2005) like in ISP2 that contained glucose as carbon source which eased the growth of unfavorable organisms too.

Presence of Cycloheximide was presumed to eliminate the growth of fungus. The slow growing colonies that appeared glabrous or chalky, heaped, folded and with aerial and substrate mycelia of different colors were thought to be putative *Streptomycetes* (Rodríguez *et. al.*, 2006) and further streaked in ISP2 and/or ISP4 for pure culture isolation. A total number of 25 putative *Streptomyces* strains were isolated from 10 different soil samples. The isolates were characterized as shown (Table 2) as recommended by Shirling and Gottlieb and were considered different based upon their colors, morphology, and appearances (Shirling and Gottlieb, 1966).

ISP4 medium was found to be more selective than ISP2 for the isolation of putative *Streptomyces* and considered better in terms of lesser contamination which is mostly probably due to the more complex carbon, nitrogen, sulfur and other nutrient sources in ISP4 (Appendix 8.4). However, some putative *Streptomyces* strains isolated from ISP2 did not grow on ISP4 so, both media were used for isolation of putative *Streptomyces.*

Contamination of fungal isolates and/or other bacterial isolates was still a major issue despite the use of an antifungal agent. The pre-treatment protocols could be developed so as to reduce undesired organisms and bacterial load in the inoculums used during culture. Also, the growth media could be modified to be suitable for *Streptomyces* growth while unfavorable for others to grow using alternate carbon, nitrogen or sulphur sources.

Table 2: Morphological characteristics of some isolates

S.N	Soil sample	Media used	Isolate	Colour of substrate mycelium	Colour of aerial mycelium	Morphology
1	Panchase	ISP2	D1	Brown	Gray	Smooth, Powdery
2	Panchase	ISP2	D2	Yellow	Creamy	Chalk like, smooth
3	Tribhuwan University	ISP4	TU2	Yellow	White	Chalk like, smooth
4	Tribhuwan University	ISP4	TU3	Yellow	White	Chalk like, smooth
5	Kanchanpur	ISP4	KA6	Yellowish	White	Smooth, chalky
6	Kanchanpur	ISP4	KA8	Yellowish	White	Smooth, powdery
7	Kanchanpur	ISP4	KA10	Light black	Dark black	Smooth, powdery
8	Phulchowki 2	ISP2	PUb1	Black	Blue	Smooth, powdery
9	Phulchowki 2	ISP4	PUb2	Yellow	White	Smooth, powdery
10	Ghodagaun	ISP2	GH2	Brown	White	Concave, powdery,rough
11	Ghodagaun	ISP2	GH3	Yellow	White	Smooth, chalky

4.2 Secondary metabolite production

As isatin (Rehman *et. al.*, 1997) and indirubin (Al-Dhabi *et. al.*, 2012) (both colored compounds) have been reported to be antimicrobials, all the isolated strains with colored colonies in one of the two growth media used for isolation were subjected to

secondary metabolite production in ISP2 broth. The strains that produced colorful extracts were presumed to be depicting the presence of secondary metabolites with potential antimicrobials. All the strains were grown on the culture medium with initial pH 7 and incubated for 7 days at 30 °C with vigorous shaking at 150 rpm.

Optimization for maximizing biomass and secondary metabolite production of each isolated species were not done in this research work because of time and resource constraints. The main drawback here is the inability of the *Streptomyces* strains to produce the secondary metabolites to its maximum potential and thereby undesired results were observed during antimicrobial susceptibility testing. Thus, structural similarity of the isolated strains to the reference strains with available optimized growth conditions for biomass and bioactive metabolite production could be used to design a growth medium for secondary metabolite production for each of the experimental putative *Streptomyces* strains.

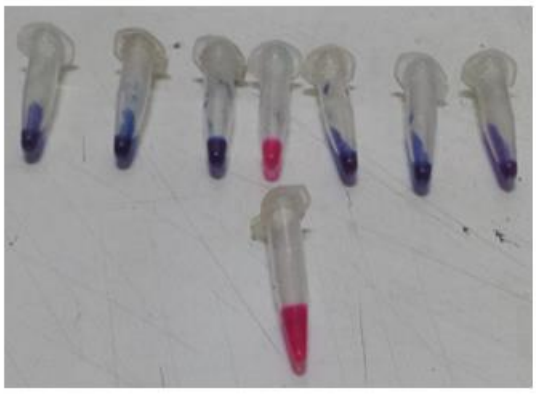
Also, the type and concentration of the components of the growth media, especially the carbon sources, could be manipulated since various reports have explained the negative carbon catabolite effects on the production of bioactive secondary metabolites (Ruiz *et. al.*, 2010).

The secondary metabolites thus produced in the growth media were extracted using ethyl acetate as a solvent, because of time constraint in this case as well, unable to look for a good solvent for extraction which could vary as per the cultures (El-Naggar *et. al.*, 2017). Because of the medium polarity and minimum toxicity on test strains, Ethyl acetate was considered a suitable solvent, also due to its ability to extract many polar as well as non-polar biological compounds.

4.3 Antimicrobial susceptibility testing

4.3.1 Resazurin antimicrobial assay

Antimicrobial disc susceptibility testing of a large number of *Streptomyces* extracts against a number of pathogens would take a considerable amount of time, labor and resources and demands robust alternative for these tests. Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). It is used as an oxidation-reduction indicator in cell viability assays for both aerobic and anaerobic respiration (Chen *et. al.*, 2015). Thus, Resazurin antimicrobial assay was used as a pre-screening tool for the extracts with antimicrobial potential. Initially the resazurin test was performed to whether it could be used for screening of antimicrobials from putative *Streptomycetes* isolates.



Red colour indicates presence of bacterial growth, Negative control
Blue colour indicates absence of bacterial growth

Figure 13a: Preliminary tests to check Resazurin antimicrobial assay.

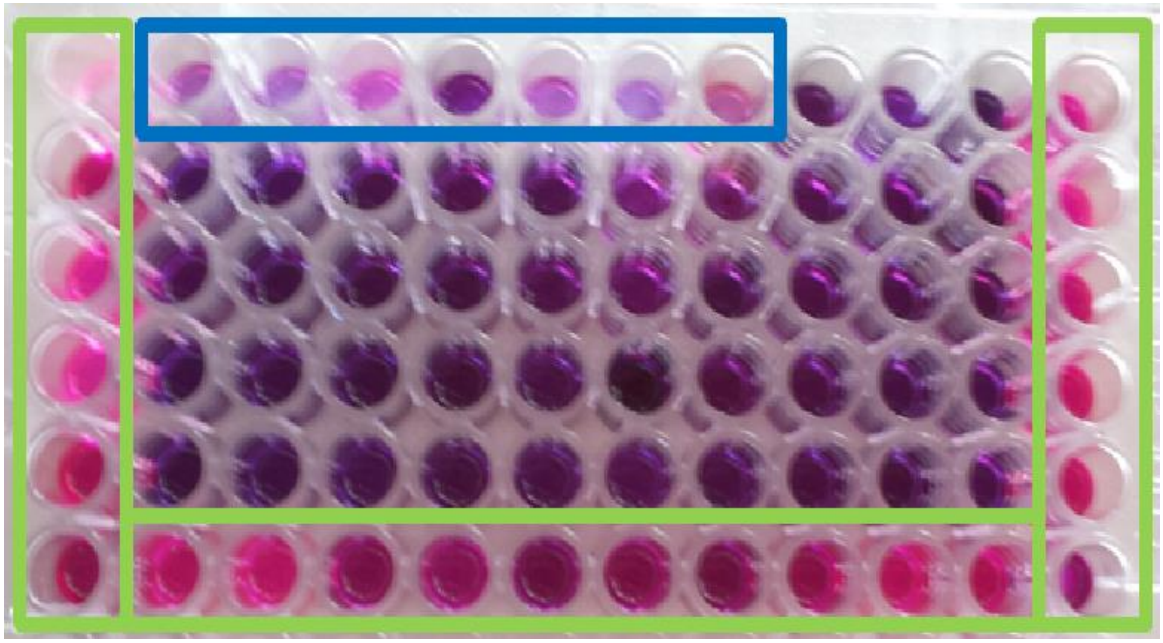


Figure 13b: Resazurin antimicrobial assay of putative Streptomyces extracts against S. aureus after 3hrs incubation at 37°C.

Pink colored (Green boxes) - negative control,

Light violet (Blue box) – AST negative and dark violet wells – AST positive

Figure 13a shows the preliminary resazurin antimicrobial assay so as to optimize the assay and check whether it functions or not. Figure 13b shows the effects of various *Streptomyces* extracts against *S. aureus*. Out of 25 extracts, 20 were found to inhibit *S. aureus*, 16 inhibited *P. aeruginosa* and 10 inhibited *P. aeruginosa* PA76, a carbapenem resistant strain. Inhibition all of the three test organisms were shown by 7 of the extracts. This clearly showed the antimicrobial potential against both Gram positive and

Gram negative strains and also a multi-drug resistant (MDR) strain. This could probably be due to the presence of broad spectrum antibiotics in the extracts produced by the respective putative *Streptomyces* strains. The inhibition could be novel in its mechanism of action since these also inhibited the carbapenem resistant strains. Further, all the experiments were repeated twice to verify the obtained results, which showed the same results. These extracts producers could be the focus of further research.

4.3.2 Disc diffusion antimicrobial assay

The disk diffusion antimicrobial assays (Balouiri *et. al.*, 2016) were also performed with the extracts exhibiting promising antimicrobials. However, contrary to resazurin assay, most extracts showed non-significant results for standard AST and only a few showed positive antimicrobial results but with inadequate zones of inhibition in the assay plates. Either the produced metabolites were in small amount so on diffusion in agar it got scattered and could not inhibit the growth of cells Or, this could be due to the loss of antimicrobial properties of the extracts as a result of decomposition of bioactive compounds (Ranglová *et. al.*, 2015) since extracts were cryopreserved for a long time at 4°C prior to Antimicrobial susceptibility testing. So, it could be concluded that the antimicrobial susceptibility tests with the extracts should be performed as soon as possible without long storage and could be stored at lower temperatures than 4°C to prevent degradation until further use.

This traditional drug discovery process is quite laborious and expensive. So, computational tools and systems biology has been the light to address present antibiotic crisis which can be used for the novel drug target identification and consequently the lead molecule identification.

4.4 Drug target protein Selection

The increasing MDR resistance in a number of pathogens and emergence of both carbapenem and colistin resistant (Ah *et. al.*, 2014; Potter *et. al.*, 2016) MDR bugs (one sample has been detected in poultry sample of Nepal, ongoing research) has now choked any available antibiotics to combat these pathogens. This demands faster discovery of novel drug targets and lead molecules against these. Thus, lead molecules with multiple target proteins in a single pathogen and with a common target in multiple pathogens are highly sought (Bolognesi, 2013) to develop a broad spectrum drug.

Simultaneously, the strategy has to be designed for preventing easy resistance development against these new drugs. Developing resistance in multiple targets at once could be evolutionarily challenging for any pathogens and probably impossible for the bacteria to survive against such developed drugs. Hence, genome level sequence alignment of major pathogens could give common new lead target proteins for

screening of lead inhibitor molecules of this protein. From this, the respective metabolic pathway or other target proteins could be identified based on protein-protein interaction databases.

4.4.1 Mauve analysis results

In order to develop drug that could have broad spectrum effects among different bacteria the conserved DNA alignment potential of a multiple sequence alignment software, MAUVE(Darling *et. al.*, 2004), was extended for whole genome sequences alignment of WHO priority list of nine pathogens (Lawe-Davies and Bennett, 2017). The genomic sequences of the pathogens in the priority lists of WHO were retrieved from NCBI to identify the common genes in all the pathogens. *Acinetobacter baumannii* strain 1656-2 (Park *et. al.*, 2011), *Campylobacter coli* 15-537360 (Pearson *et. al.*, 2013), *Enterococcus faecium* Aus0004 (Lam *et. al.*, 2012), *Helicobacter pylori* 2017 (Avasthi *et. al.*, 2011), *Klebsiella pneumoniae* subsp. pneumoniae 1084 (Lin *et. al.*, 2012), *Neisseria gonorrhoeae* FA 1090 (https://www.ncbi.nlm.nih.gov/nuccore/AE004969) *P.aeruginosa* B136-33 (https://www.ncbi.nlm.nih.gov/nuccore/NC_020912.1) *Staphylococcus aureus* 04-02981 (https://www.ncbi.nlm.nih.gov/nuccore/NC_017340.1) and *Salmonella typhimurium* (Silva *et. al.*, 2016) along with *Mycobacterium tuberculosis* (https://www.ncbi.nlm.nih.gov/nuccore/NC_021054.1) whole genome sequences were aligned for putative lead target protein identification against which drugs could be developed.

Figure 14: Multiple sequence alignment in MAUVE, showing metK as a common gene in all 9 pathogen's genomes aligned (data for remaining 5 not shown)

These drugs when developed could act against most of the pathogens listed. The presence of aligned genes exhibited similar color (Figure 14). Although computational curing method and algorithm development could reduce time for identification of common genes, such as in two or three or four or five pathogens only as potential lead target proteins but at this stage it is manual curing.

Table 3: Putative lead target proteins identified as common in whole genome sequence alignment of WHO priority list pathogens (Lawe-Davies and Bennett, 2017) and M. tuberculosis.

Protein type	Gene: Protein Name (Reference)
Ribosomal proteins (30s)	*rps*C: 30s ribosomal protein subunit S3 (PMID: P0A7V3); *rps*E: ribosomal protein S5 (PMID: P0A7W1); *rps*H: 30s ribosomal protein S8 (PMID: P0A7W7); *rps*N: 30s ribosomal protein S14 (PMID: P0AG59); *rps*Q: 30s ribosomal protein subunit S17 (PMID: P0AG63);
Ribosomal proteins (50s)	*rpl*C: 50s ribosomal protein L5 (PMID: 6760192); *rpl*F: 50s ribosomal protein L6 (PMID: P0AG55) ; *rpl*N: ribosomal protein L14(PMID: P0ADY3); *rpl*O: 50s ribosomal protein L15 (PMID: 3298242) but not aligned in *M. tuberculosis*; *rpl*P: 50s ribosomal protein subunit L16 (PubMed: 8524654); *rpl*R: 50s ribosomal protein L18 (PMID: 354687); *rpl*X: ribosomal protein L24 (PMID: 357435); *rpm*C: 50s ribosomal protein subunit L29 (PMC 327435); *rpm*D: 50s ribosomal protein L30 (Pubmed 3297162);
Involved in ATP synthesis	*atp*A: ATP synthase subunit alpha (EC:3.6.3.14); *atp*G: ATP synthase gamma chain (Uniprot ID: P95788); *atp*D: ATP synthase subunit beta (EC:3.6.3.14).
DNA directed RNA polymerase	*rpo*B: DNA-directed RNA polymerase, beta subunit (PMID: 1646077); *rpo*C: DNA-directed RNA polymerase, beta subunit (PMID: 1646077).
Chaperon (absent in *M. tuberculosis*)	*Dna*k: chaperone protein Dnak (Uniprot ID: P9WMJ9); *clp*B: chaperone protein ClpB (PMC1819559, PMID:12624113, PMC1821349).
Elongation factor	*fus*A: Elongation factor G (Uniprot ID: P0A6M8)
Protein translocator	*sec*Y: Protein translocase subunit (Pubmed 1531482, Pubmed 7650029).
Thiol assimilation	*MetK*: methionine adenosyl transferase (PMID: 6251075).

Curing the alignment revealed twenty four common genes found in most of the strains with diverse roles. Most of the genes (Table 3) were ribosomal proteins (14 proteins), involved in ATP synthesis (3 proteins), DNA directed RNA polymerase (2 proteins), chaperon (2 proteins), elongation factor (1 protein), protein translocator (1 protein), involved in thiol assimilation (1 protein).

The MAUVE results that showed *metK* as one of the probable therapeutic targets and was taken as a reference on our search for other therapeutic targets. In earlier works on *S. typhimurium* LT2, *metK* was found to be essential. The *metK* gene codes for the formation of S-adenosylmethionine (SAM) from ATP and methionine as substrates (http://www.uniprot.org/uniprot/P0A817). The product of *metK*, SAM is utilized by three major metabolic pathways: transmethylation, transsulfuration, and polyamine synthesis making SAM, an important molecule in normal cell functioning and survival (Lu, 2000) making it a critical metabolite for cellular functions.

Transmethylation reactions include the methylation of a variety of substrates including DNA, phospholipids and proteins involved in various processes ranging from gene expression to membrane fluidity (Thong *et. al.*, 1987) and the focus of the present study. Transsulfuration reactions include the conversion of Sulphur group of SAM via a series of enzymatic steps to cysteine (Aitken *et. al.*, 2011), a precursor of glutathione (Penttilä, 1990) and taurine (Bin *et. al.*, 2017) and a major cellular anti-oxidant (Kerksick and Willoughby, 2005). Also, polyamines are necessary for normal growth (Li and Tian, 2016). All these make SAM and thus MetK an important drug target.

In addition, the inhibitor of *metK* could potentially work as inhibitor to genes involved in ATP biosynthesis and DNA directed RNA polymerases due to presence of adenosine moiety in ATP and SAM. The other ribosomal proteins could be lead target proteins which could be further explored. But, with FBA analysis using COBRA tool, these proteins could not be explored in the present study. Thus, among the common genes, *metK*, was taken as the potential target present in all these MDR strains, which was subjected to gene essentiality studies.

4.4.2 Active binding site visualization

In addition, SAM is primary methyl donor in multiple reactions including, corrin ring methylation (Roper *et. al.*, 2000), RNA methylation (Motorin and Helm, 2011), DNA methylation (Sánchez-Romero *et. al.*, 2015) and these steps are taken as lead targets. Hence, MetK was taken as the lead protein and its interacting proteins in String of *P. aeruginosa* (Uniprot: Q9I5Z0) are found to be critical in cellular responses (https://string-db.org/network/208964.PA0546). Protein sequence alignment of MetK from these pathogens were done to identify similarity in active sites.

Figure 15: Clustal Omega alignment of Metk protein from eight pathogens.

Green – denotes binding site; Purple – denotes metal binding

Upon protein alignment, the active binding sites in MetK were found same (conserved) in all the pathogens under study. The clustal Omega results of MetK proteins of *Acinetobacter baumannii BIDMC 57* (A0A2C9TDB1), *Campylobacter coli* (A0A0Q2J0S7*), Enterococcus faecium* (A0A1A7T313*), Helicobacter pylori* (P56460*), Klebsiella pneumoniae subsp. pneumoniae (strain ATCC700721 / MGH 78578* (A6TDV1)*, Neisseria gonorrhoeae (strain ATCC 700825 / FA 1090)*(Q5FAC0)*, Pseudomonas aeruginosa* (A0A072ZEM3*), Staphylococcus aureus (strain USA300 / TCH1516*)(A8Z4L2) and *Salmonella typhi* (P66765) show the common active binding sites in all these aligned organisms (Figure 14).

This indicates that this protein could be a lead protein target to develop drug. Despite differences in their length, all these MetK proteins had common active sites and metal binding sites. Hence, a molecule that could be competitive inhibitor of this protein may inhibit methyl transfer reactions and be a drug candidate to tackle different Gram negative MDR strains that are highly prioritized pathogens of WHO against which new drug is sought. Further analysis for gene essentiality for pathogen survival could reveal whether this is the lead target protein.

4.4.3 Gene essentiality analysis and its therapeutical applications

Essential genes are those genes which are crucial for the survival of an organism. The essential genes represent potential novel drug targets for numerous antimicrobials (Duffield *et. al.*, 2010; Lu *et. al.*, 2014). The main objective of gene essentiality would be to target a cellular function of a pathogen under study that is universally essential for that pathogen under most, if not all, environments so that the potential drugs

developed against it will be effective against various clinical isolates of that particular pathogen and in any possible body parts in the host.

In this research, we searched for broad-spectrum compounds focusing on conserved essential genes across species and also multiple essential genes within a species at the same time as a means to prevent the possible resistance development against the newly developed drug with the passage of time. The basic idea is to search for multiple drug targets against a pathogen which are most, if not all, conserved in all other pathogens under study.

In earlier works SAM has been found to be an essential metabolite thus making *metK* gene essential. Moreover, Quorum sensing is one of the major causes of resistance in pathogens which utilize autoinducers which inturn utililze SAM as a substrate (Yunzhou *et. al.*, 2011). Also, it controls biofilm formation and virulence in bacteria (Rutherford and Bassler, 2012),.

Thus, gene essentiality test for *metK* gene and genes involved in Vitamin B12 biosynthesis, corrin contraction pathway were done to identify as a lead target for methyl transfer inhibitor drug development since this corrin ring contraction is done through methylation of different carbons and the enzymes utilize SAM. Apart from SAM, vitamin B12 also is involved in methyl transfer reactions (Ragsdale, 2008). The test was performed for various pathogens under study. Because COBRA tool requires metabolites for analysis and *dam* and *trmD* though use SAM as substrate, their products are not metabolites, so these were excluded. Out of nine pathogens, only four functioning genome-scale models were available till date and gene essentiality in these four pathogens was performed. It was found that (Table 4) *metK* and *cobA* deletion could be fatal in all these test organisms. This result clearly indicated *metK* and *cobA* genes along with SAM riboswitch as potential therapeutic targets against which lead molecules could be searched for.

Literature search further verified the essentiality of *metK* in many different pathogens (Yuhong and Newman, 2002; Walker *et. al.*, 2016). Also, the lack of reports about SAM transporters in any of the mentioned target pathogens makes this a better target. So, including MetK, some other SAM utilizing proteins, namely CysG, Dam (DNA adenosine methylase), CobA (uroporphyrinogen-III methyl transferase), TrmD (tRNA (guanine-N(1)-)-methyltransferase) and SAM riboswitch were taken as potential drug targets against which virtual screening of compounds were done.

Table 4: Results of gene deletion analysis on various models

(✓ = Growth, × = no growth)

Organism / Gene	*Pseudomonas aeruginosa* (iPAU1129)		*Salmonella* STM_v1.0		*Staph.aureus* iSB619		*Helicobacter pylori* iIT341	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
MetK	×	×	×	×	×	×	×	×
cobA	✓	×	×	×	×	×	N/A	N/A
cobI / cbiL	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobG	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobJ / cbiH	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobM / cbiF	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobF / cbiD	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobK / cbiJ	✓	×	✓	✓	N/A	N/A	N/A	N/A
cobL,cbiEandcbiT	✓	×	✓	✓	N/A	N/A	N/A	N/A
Uroporphyrinogen III synthase	×	×	×	×	×	×	✓	×

4.4.4 *metK* as an antibacterial target

The gene deletion studies depicted that *metK* is among the common essential genes among all the listed pathogens which makes this an interesting drug target. *MetK*, S-adenosylmethionine synthase, catalyzes the reaction between methionine and ATP to form S-adenosylmethionine (SAM) which is a common co-substrate involved in various reactions including transsulfurations, aminopropylations and methyl group transfers to nucleic acids, proteins, lipids and secondary metabolites.

4.4.5 Vitamin B12 biosynthesis pathway as potential therapeutic target

Cobalamin (vitamin B12) is a structurally complex cofactor, consisting of a modified tetrapyrrole with a centrally chelated cobalt. Cobalamin is most commonly

found in two biologically active forms: methylcobalamin and adenosylcobalamin. Most prokaryotes as well as animals have cobalamin-dependent enzymes as important aspects of their metabolic pathways, whereas plants and fungi do not appear to use it.

In bacteria and archaea, these enzymes include diol dehydratase (Toraya, 2002), ethanolamine ammonia lyase – a key element of glycerophospholipid metabolism (Mori *et. al.*, 2004), methionine synthase - a part of SAM biosynthesis and regeneration cycle (Gruber and Kratky, 2006), ribonucleotide reductase that catalyzes the formation of deoxyribonucleotides from ribonucleotides (Taga and Walker, 2010) and glutamate and methylmalonyl-CoA mutases involved in various catabolic or biosynthetic pathways (Gruber and Kratky, 2002; Takahashi *et. al.*, 2012). Some of these enzymes have been reported to be essential for bacterial survival (Savvi *et. al.*, 2008; Torrents, 2014) and the absence of some prevent bacterial pathogenesis (Garsin, 2010). All these add up to the cobalamin being a probable therapeutic target.

Gene deletion studies (Table 4) showed only *cobA* among all other genes involved in corrin ring contraction through methylation in cobalamin biosynthesis pathway as an essential gene in many bacteria. Thus CobA protein could be used as a potential therapeutic target. Hence, molecular docking was done for these narrowed proteins.

4.5 Molecular docking

For *in silico* screening of putative drug candidates MetK, CobA and SAM-I riboswitch were chosen as potential drug targets. The crystal structures of these proteins and RNA were not available for all the pathogens under study so the crystal structure of MetK (PDB id: 5T8S) isolated from *Neisseria gonorrhoea*, CobA (PDB id: 1V9A) isolated from *Thermus thermophilus* and SAM-I riboswitch (PDB id: 4KQY) isolated from *Bacillus subtilis* were used. The active binding sites of MetK proteins were same for all the pathogens under study (Figure 14), thus, these available structures could be considered for docking. However due to less than 50% similarity among the amino acid (AA) sequences of MetK of *Neisseria gonorrhoea* with other pathogens, the 3D structure of MetK for other pathogens were predicted from their primary AA sequences and the docking results were compared.

4.5.1 Protein Tertiary structure analysis:

The target proteins were downloaded from their respective RCSB protein data bank sites. For those whose crystal structures were not available in RCSB PDB and SCOP databases, primary AA sequences were retrieved from NCBI and were used to predict their tertiary structures using various web-based tools including CPHmodel, Phyre2, (PS)2v2, RaptorX and Swiss-Model.

Using different available web based tools the Z score of the predicted 3D structure for MetK proteins of different organisms were calculated. Based on the Z-score following tools were found to be reasonable for predicting 3D structure of respective MetK protein (Table 6).

Table 5: Z-score and number of amino acids indicating accuracy and completeness of predicted structure

Name of server	Z score	AA no.
CPH model	-8.35	383
Phyre2	-8.14	384
Swiss model	-8.23	383
Ps2v2	-8.08	384
RaptorX	-8.29	384

The accuracy of the predicted structures was a key to successful molecular docking strategies since it determines the effectiveness of the results from further works, for which Z-score was considered as a standard parameter of quality assessment (Wiederstein and Sippl, 2007). The quality assessment of predicted 3D-protein structures was done using ProSA-server. The Z-score of the input structure should be within the range of scores typically found for native proteins of similar size as shown in ProSA. ProSA-web is an interactive web service for the recognition of errors in both experimental and theoretical 3D models of proteins structures (Wiederstein and Sippl, 2007).

In the figure, the first plot (Figure 16a) shows the local model quality by plotting energies as a function of amino acid sequence position. Generally, positive values refer to erroneous or inaccurate parts of the input structure. Similarly, in the second plot (Figure 16b), structures from different sources (X-ray, NMR) are distinguished by different color codes as shown. The plot is used to check whether the Z-score of the query structure is within the range of scores typically found for the similar sized native proteins.

a) b)

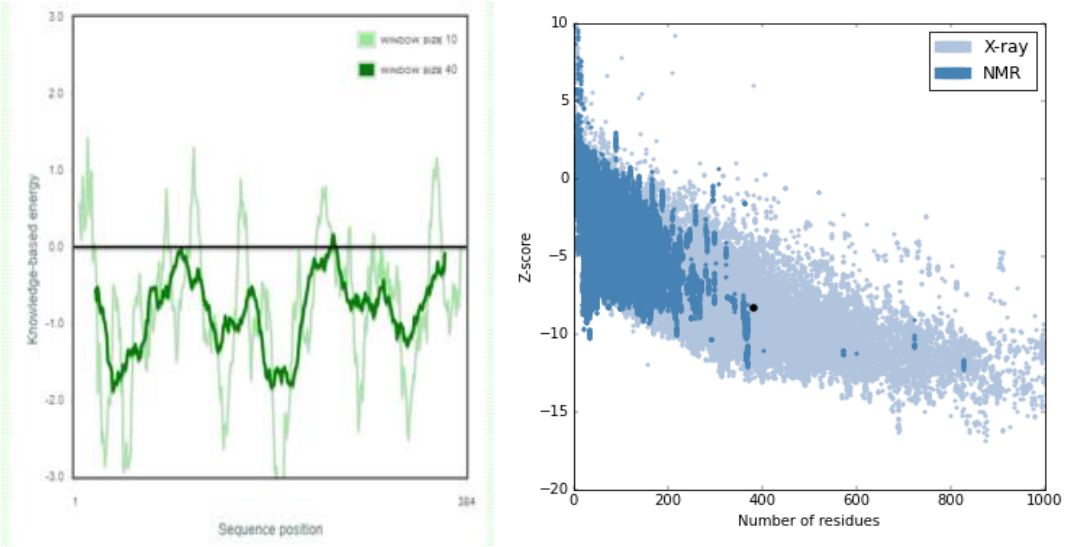


Figure 16: The Z score plots and energy plot for predicting 3D structure of a protein based on amino acids sequences.

a) Z-Score plot of model predicted by RaptorX with structures available in database.
b) Energy plot for amino acid residues for the 3D-structure of MetK of *Klebsiella pneumonia* predicted from RaptorX.

The structure predicted by RaptorX had the lowest Z-score and it also modelled the most complete structure of MetK. Thus, this was considered as the best predicted 3D structure for MetK of *Klebsiella pneumonia.* The selection of structures for metK of 8 other pathogens were done as mentioned in Table 6.

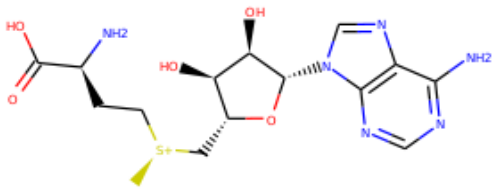
Table 6: Servers producing the most accurate 3D structures of metK

Organisms	Name of server
Acinetobacter baumanii	Phyre2
Campylobacter coli	RaptorX
Enterococcus faecium	Phyre2
Helicobacter pylori	Phyre2
Pseudomonas aeruginosa	Phyre2
Staphylococcus aureus	(PS)2v2
Salmonella enterica serovar *Typhi*	Phyre2

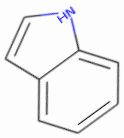
4.5.2 Ligand database preparation

Another vital process prior to molecular docking is to prepare a ligand database which could contain potential leads against the target proteins. The adenosyl moiety of SAM and ATP binding domain present in kinases (Villamor *et. al.*, 2013) probably suggests kinase inhibitors as potential inhibitors of these proteins that biosynthesize or utilize SAM. Protein Kinase inhibitors represent an important class of targeted therapeutic agents, particularly as anticancer drugs (Grant, 2009). Indole is reported to be metabolized by human cytochrome P450 2A6 (Gillam *et. al.*, 2000) and the source of indole could be from tryptophan metabolism by gut microflora. This indicates that indole could be easily transported in human through gut suggesting indoles are metabolized in humans thus indicating these could not pose toxicity (Banoglu *et. al.*, 2001). In addition, indole has been suggested to be pharmaceutical scaffold for drug development (Suenkel *et. al.*, 2013).

Also, its metabolized derivative Indirubin has been reported to be kinase inhibitor (Wu *et. al.*, 2005) and some indole derivatives also have been reported to be kinase inhibitors in multiple instances (Kiliç *et. al.*, 2009; Ölgen *et. al.*, 2011). The close structural proximity of indole ring to adenosyl moiety of SAM (Figure 17) further pushes indole derivatives to be probable candidates against SAM binding pocket of Metk protein. In addition, earlier works on *E. coli* Metk protein using ligands from NCI database II (based on the hypothesis that some of the anticancer agents could be kinase inhibitors and they could act on SAM binding pocket) revealed two compounds in which one had indole moiety (unpublished data, Rimal *et. al.,* 2016). Thus, it was presumed that indole derivatives could be the ligand sources for virtual screening.



S-adenosyl methionine (SAM)



Indole

Figure 17: Chemical structure of SAM and indole.

Thus, Indole derivatives were searched in ZINC database, a free database of commercially available compounds. The ligands were prepared by energy minimization using OpenBabel: An open chemical toolbox (O'Boyle *et. al.*, 2011). A total of 715 dockable Indole derivatives including marine indoles (Netz and Opatz, 2015) was downloaded from ZINC database.

4.5.3 *In-silico* ADME/Tox tests:

Once the ligand library was made, all the ligands could be directly used for docking. However, the chemicals may not pass ADME and toxicity test. Thus, performing ADME/T could be used in earlier step. The primary reason for lead molecules not being able to pass the clinical trials is their inability to reach the target and perform its predicted function, and also the toxicity issues (Hughes *et al.*, 2011). Thus, ADMET and pharmacokinetic properties evaluation in the early stages of drug discovery seem to be a wiser choice. The toxic profiles and druglikeness were predicted using OSIRIS.

The selection criteria include:

- Mol weight – 200 to 500 Daltons
- cLogP – -3 to 6

The clogP similarity of a compound, which is the logarithm of its partition coefficient between n-octanol and water $\log(c_{octanol}/c_{water})$, is well established measure of the compound's hydrophilicity. Low hydrophilicities and therefore high logP values cause poor absorption or permeation. It has been shown for compounds to have a reasonable probability of being well absorbed, their clogP similarity must not be greater than 5.0.

- cLogS – greater than -4 i.e. -4 to -2

The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. Typically, a low solubility goes along with a bad absorption and therefore the general aim is to avoid poorly soluble compounds.

- Hydrogen bond donors – 0 to 5
- Hydrogen bond acceptors – 0 to 10 (Lipinski, 2004)
- Topological Polar Surface Area (tPSA) – 0 to 140
- Rotatable bonds – 10 or less (Veber *et. al.*, 2002)
- Druglikeness – Positive value

OSIRIS calculates various drug relevant properties like molecular weight, cLogP, cLogS, Druglikeness, and toxicities like mutagenicity, tumorigenicity, reproductive effects and irritant effects in the lead molecules on the basis of functional groups present in their structures (Sander *et. al.*, 2015). ADMET filters narrowed these 715 indole derivative ligands down to 102 ligands (Appendix 8.5).

4.5.4 Identification of Binding Site

The ligand binding sites were identified from the crystal structure of S-adenosyl methionine synthase (MetK) of *N. gonorrhoea* (PDB id: 5T8S) available in RCSB protein data bank. For the predicted 3D-structures of MetK for which crystal structures have not yet been elucidated, 3DLigandSite, a web server that superimposes the ligands bound to the structures similar to the query and thus predicts the binding site (Wass *et. al.*, 2010), was used to predict the ligand-binding sites.

All the AA residues around the binding site as shown, (Table 7) are marked to create a site for molecular docking.

Table 7: Predicted binding sites and heterogens present as predicted by 3DligandSite in MetK *of Helicobacter pylori predicted via Phyre2 webserver*

Predicted Binding Site			
Residue	Amino acid	Contact	Av. distance
42	ALA	5	0.00
57	GLU	5	0.00
59	LYS	5	0.00
118	GLY	4	0.55
119	ASP	5	0.02
266	LYS	3	0.04
270	LYS	5	0.02

Heterogens present in Predicted Binding Site		
Heterogen	Count	Source structures
ADP	2	1mxb_A,1o92_B
SAM	2	1rg9_D,1p7l_C
MG	5	1xra_A,1mxc_A, 1mxb_A,1mxa_A ,1xrb_A
ATP	1	1o93_B

4.5.5 Virtual Screening of the Indole derivatives

Docking was performed using AutoDock Vina in Virtual Screening Software, Pyrx (https://pyrx.sourceforge.io/) with an easy-to-use user interface. The target proteins and RNA (SAM riboswitch; Appendix 8.6) were docked to screen ligands with multiple targets. As it is reported that the higher the negative value, the stronger is the binding of the ligand in the target (Dallakyan and Olson, 2015), the binding energy showing greater negative value was taken in consideration. Out of 102 ligands that passed ADME/T test, 58 ligands exhibited higher binding energy (BE) than the native ligand SAM towards metK in *N. gonorrhoea* (PDB id: 5T8S; Appendix 8.7). This indicated that these ligands could potentially block MetK in releasing the SAM from active pocket thereby inhibiting SAM biosynthesis.

Since most of the proteins exhibited similar binding AA site in alignment (Figure 15), it is presumed that available 3D structure of *N. gonorrhoea* and 3D structure predicted from different web based tools could be used for docking. All these 58 ligands were further

docked against the SAM binding pocket of all other pathogens under study using the predicted 3D structures from various web based tools.

Table 8: Druglike indole derivatives with higher B.E than SAM (probable metK inhibitors)

Zinc Ids	*N. gonorrhoea* -8.7*	*A. baumannii* -7.2*	*C. coli* -6.4*	*K. pneumoniae* -6.2*	*E. faecium* -5.6*	*H. pylori* - 4.7 *	*P. aeruginosa* -6*	*S. aureus* - 6.1*	*S. typhii* - 6.1 *
ZINC000 0585351 34	-10.3	-7.8	-9.3	-7.4	-5.8	-5.2	-7.7	-7	-6.5
ZINC025 60263	-7.2	-7.8	-7.6	-6.3	-5.3	-7	-6.2	-7.2	-7.2
ZINC048 99565	-10.4	-7.7	-8.3	-7.4	-6.9	-5.3	-6.9	-6.7	-7.3
ZINC060 96559	-10.4	-7.6	-8.2	-8.7	-7	-5.3	-7.3	-6.9	-6.9
ZINC152 19763	-9.5	-7.5	-9.8	-8.3	-7.1	-6.1	-7.8	-6.9	-7.5
ZINC491 69056	-10	-7.5	-8.1	-7.7	-7.5	-5.1	-7.3	-6.8	-7
ZINC491 69727	-11.1	-7.5	-7.2	-6.8	-6.9	-5	-7.3	-7.7	-6.7
ZINC491 71024	-10.8	-7.6	-7.9	-7.4	-6.9	-5.6	-7.8	-6.9	-7
ZINC491 71033	-10	-7.8	-8.1	-7.7	-6.5	-5.5	-7.7	-6.9	-6.8

*Binding energy of native ligand SAM (Binding energy as KJ/mol).

Out of 58 ligands with higher energy than SAM in *N. gonorrhoea,* only nine ligands were found to have higher BE than SAM (native ligand) in the respective MetK proteins of all the 8 other pathogens (Table 8) indicating that these molecules could potentially inhibit MetK activity in all the pathogens under the study.

4.5.6 Multi-target potential of the lead molecules

Since SAM is utilized in several cellular functions, the focus was mainly in methyl transfer reactions. Mainly the focus was on CobA for preventing cobalamin biosynthesis which is required for lipid biosynthesis (Fidanza and Audisio, 1982) thus cell membrane biosynthesis could be prevented (Dowhan *et. al.*, 2008). In addition, bacterial DNA methylation by Dam protein was also of interest as DNA methylation is responsible for DNA replication (Boye and Løbner-Olesen, 1990).

Table 9: Druglike indole derivatives with probable broad spectrum antimicrobial activities

Ligands	Zinc Ids	Metk	CysG	CobA	Dam	TrmD	SAM-I riboswitch
		-8.7*	-7.5*	-8.3*	-8.9*	-7.3*	-10.2*
3-[(4-hydroxyphenyl)methyl]-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione	ZINC04899565	-10.4	-9.4	-9.5	-10.1	-10	-10.5
3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione	ZINC06096559	-10.4	-8.5	-9	-9.8	-8.5	-10.8
3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione	ZINC06096622	-10.8	-9.3	-9.8	-10.7	-9	-10.5
(1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione	ZINC15219763	-9.5	-9.1	-9.9	-11.1	-9.9	-11
3-((4-(1H-indol-3-ylmethyl)-1-piperazinyl)methyl)-1H-indole	ZINC19909549	-10.6	-9	-8.7	-9.7	-8.1	-10.5
2-[(3S)-1-(1H-indol-3-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole	ZINC49169056	-10	-9.5	-9.2	-10.9	-9.4	-10.4
1-[(3S)-3-[5-(1H-indol-3-ylmethyl)-1,3,4-oxadiazol-2-yl]pyrrolidin-1-yl]ethanone	ZINC49171024	-10.8	-9.1	-8.8	-9.9	-9	-11.1
1-Methyl-3,4-bis(3-indolyl)maleimide	ZINC02560263	-10.1	-9.7	-8.9	-10	-7.4	-10.9

*- Binding energy of the native ligands

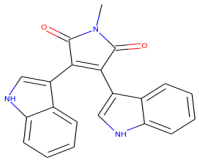
This Dam methylation varies with human in that it methylates adenine in DNA contrary to human cytosine (Kumar *et. al.*, 1994). In addition, Dam methylation is also responsible for the mRNA transcription (Low *et. al.*, 2001). Thus, interfering with Dam methylation could block DNA replication and/or mRNA transcription and be lethal to bacterial survival. Moreover, SAM-I riboswitch was also of interest as the drug target for preventing translation of the protein (Batey, 2011) even if the mRNA is transcribed. Furthermore, hampering TrmD activity that methylates $tRNA^{PRO}$ essential for growth

(O'Dwyer *et. al.*, 2004) to induce +1 frameshift during peptide bond elongation (Gamper *et. al.*, 2015) for defunct protein folding even if the mRNA is translated.

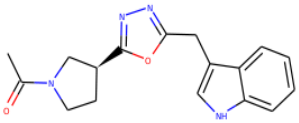
Thus, the target was to prevent DNA replication, mRNA translation, prevent peptide elongation even if mRNA is transcribed, and even if the peptide elongation occurs, induce frameshift mutation to make defunct protein and finally disrupt cell membrane to lyse the cells.

The 58 ligands selected from docking results in MetK (5T8S) were also docked against the available structures in rcsb.org of other targets, CysG of *Salmonella typhimurium* (strain LT2/ATCC 700720), CobA of *Thermus thermophilus* ATCC 27634, Dam of *Streptococcus pneumoniae*, TrmD of *Haemophilus influenza* and SAM-I riboswitch of *B. subtilis*. Out of these, only 8 showed higher B.E than the native ligands in the respective proteins (Table 9).

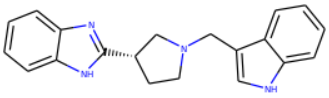
Out of these eight indole derivatives, six are also seen to inhibit MetK in all the 9 target pathogens which could potentially be developed as broad-spectrum antimicrobials with multiple targets. The molecular structures, ZINC ID and molecular name of these six indole derivatives are illustrated (Figure 18). Other indoles depending upon their binding affinity could be also used to develop new drug candidates by side chain modifications.



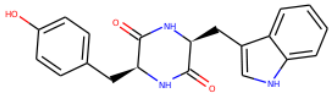
1-Methyl-3,4-bis(3-indolyl)maleimide



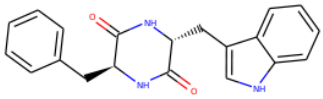
1-[(3S)-3-[5-(1H-indol-3-ylmethyl)-1,3,4-oxadiazol-2-yl]pyrrolidin-1-yl]ethanone



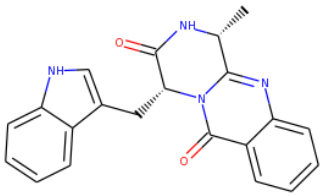
2-[(3S)-1-(1H-indol-3-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole



3-[(4-hydroxyphenyl)methyl]-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione



3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione



(1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione (Fumiquinazoline G)

Figure 18: Molecular structures of molecules that could be developed as putative antibacterial drugs.

4.5.6 Cross-reactivity with Human Homologues

Questions on cross-reactivity with human homologues of S-adenoyslmethionine synthase (Uniprot id: Q00266) could be raised as it has a 53.102 % structural similarity

(results not shown) with that of bacteria (*E.coli* taken as model organism) (Uniprot id: P0A817). Unfortunately, molecular docking of five out of six lead molecules against the SAM binding pocket of its human homologue showed these molecules as potential inhibitors of its human homologue as well (Appendix 8.8).

Nevertheless, MetK inhibitors of human could still be used as antibacterial therapeutics because of the presence of SAM transporters in humans (Agrimi *et. al.*, 2004) and the SAM requirements can be replenished from external sources. The lack of crystal structures of SAM transporters in humans constrained the molecular docking studies of possible inhibition of the transport system.

However, the people with poor liver functions could require SAM as hepatoprotective agent (Angelini *et. al.*, 2012). Moreover, ZINC02560263 (1-Methyl-3,4-bis(3-indolyl) maleimide) that had lower binding energy than SAM in human homologue (-7.3 Kcal/mol compared with SAM -8.7 Kcal/mol) could be developed as a therapeutic drug against these bacteria with minimal negative effects on humans. Thus, alternatively ZINC02560263 that showed lower affinity than the native ligand in human Metk protein but higher affinity than the native ligand (SAM) in MetK of all eight pathogens makes the ligand, 1-Methyl-3,4-bis(3-indolyl) maleimide (Figure 19) as a potential lead molecule that could be developed as a putative therapeutic drug.

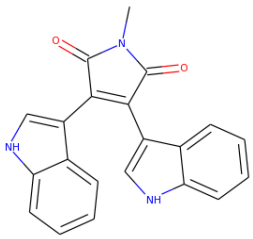
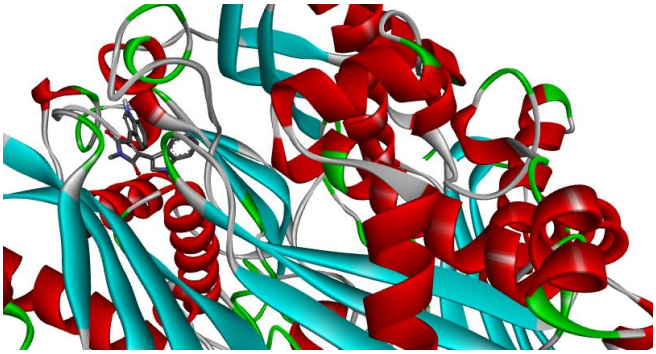


Figure 19: Molecular structure of ZINC02560263 (1-Methyl-3,4-bis(3-indolyl) maleimide)

Furthermore, ZINC02560263 (1-Methyl-3,4-bis(3-indolyl) maleimide) also known as bisindolyl malemide V is a bisindolyl maleimide (BM) derivative and has been reported to be devoid of proteinase kinase C (PKC) inhibition activities compared to other BMs suggesting that the adenosine moiety binding area could be competitively inhibited by this. It has been reported to prevent oxidative stress induced necrosis of variety of cells including neurons (Asaki *et. al.,* 2002).

The binding of ligand in 3D structure of *N. gonorrhoea* MetK and respective amino acids bound to respective amino acids compared with the SAM binding amino acids are depicted in figure 20.

A



B

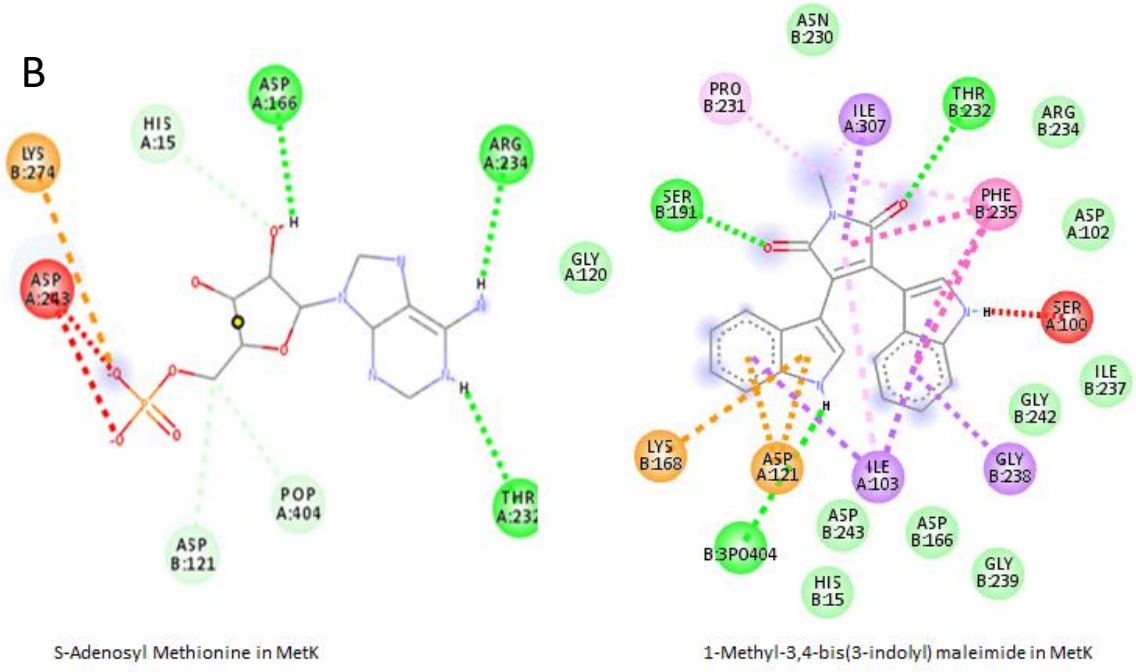


*Figure 20: ZINC02560263 at the receptor of Metk protein (*5T8S*) of N. gonorrhoea*

A: Ligand at the docked site B: 2D view of Amino acid residues interacting with the ligand, at the docked site of MetK (5T8S)

5. SUMMARY

The world is in immediate need of novel antibiotics to cope against the emerging pathogens, some being multi-drug resistant (MDR) and causative agents of numerous deaths around the globe. Computational techniques including Molecular docking were employed to screen out the ligands from huge database collection of ligands that could potentially act as competitive inhibitor against the various target proteins under study. An indole derivative 1-Methyl-3,4-bis(3-indolyl) maleimide was found to inhibit MetK in all the nine WHO priority listed pathogens and also various other potential drug targets including CysG, CobA, Dam, TrmD and SAM-I riboswitch. Also, this molecule did not affect the human homologue of Metk protein which makes this as a lead candidate with potential activity against various pathogenic bacteria and with minimal side effects on human. Previous works by Wu *et. al.* (2005) have shown the indole derivatives can be metabolized by cytochrome P450 2A6 into indirubins with more potent activity against Glycogen synthase kinase, GSK-3 β enzyme indicating the indole derivatives would not be toxic to humans thus the lead candidates could pass human trials. Molecular docking of these six lead candidates against GSK-3 β (RCSB id: 1Q5K) showed all six to bind to the receptor of GSK-3 β with higher B.E than the known GSK-3 β inhibitor N-(4-Methoxybenzoyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea (Appendix 8.9) meaning these lead indole molecules could produce better results when converted into their respective indigoids by the activity of human cytochrome P450 2A6. Similarly, the lead indole molecule from this study could itself be used as a drug candidate or could be metabolized into other indirubin derivatives with potential stronger activity against the targets. The present research suggests that computational biology could be handy in narrowing down the lead target proteins and putative drug-like molecules for efficient development of new antimicrobials. Since, the hypothesis is based on kinase inhibition potential of some of these derivatives the narrowed compound could also be investigated as anti-cancer agents.

6. CONCLUSION

Present study revealed that some of the putative *Streptomycetes* isolated could be producing antimicrobial agents but optimization of production should be developed to have higher amount of yield. However, the extracts upon storing in refrigeration (few months) did not show any zone of inhibition which either indicated that the amount in the extract was too low to exhibit inhibition in contrary to broth assay with resazurin or the product must have been degraded making the molecule unstable. Thus, a robust screening of new potential antimicrobial with multiple targets and in multiple pathogens should be developed. Modifying the application of MAUVE software that is predominantly used to identify common genomic sequences among closely related bacteria towards whole genome sequences alignment, the genes that are common in nine pathogenic bacteria were identified as potential lead target proteins. Taking essential SAM molecule in several bacterial functions and previous works on screening of anti-cancer agents based on the hypothesis that some of the anti-cancer agents could be kinase inhibitors and one of the lead molecule was an Indole derivative it was hypothesized that indole derivatives could be putative competitive inhibitors for SAM binding. In addition, indole derivatives and their metabolites have been reported to function as kinase inhibitors and metabolism of indole by cytochrome P450 2A6 indicates that indole and its metabolite are found in human, it was presumed that indole derivatives could be less toxic to human. Thus, screening of indole derivatives were done as competitive SAM inhibitors because of adenosine moiety of SAM that is derived from ATP on the hypothesis that the kinase inhibition by indole could also be competing with the adenosine binding pocket of kinases. The indole derivatives were narrowed based on their ADME/T and virtual binding energy that exhibited higher binding efficiencies than native ligand SAM. This was further expanded to multiple target proteins and SAM-I riboswitch. From these results, five compounds namely 2-[(3S)-1-(1H-indol-3-ylmethyl)pyrrolidin-3-yl]-1H-benzimidazole, 1-[(3S)-3-[5-(1H-indol-3-ylmethyl)-1,3,4-oxadiazol-2-yl]pyrrolidin-1-yl]ethanone, 3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione, (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione, and 3-[(4-hydroxyphenyl)methyl]-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione had higher binding energy than SAM in MetK, CysG, CobA, Dam, TrmD and SAM-I riboswitch. These molecules also showed higher binding energy than SAM in human SAM synthase so this might implicate some effects to human. However, SAM could be co-administered as it can be transported in human cells but cannot be transported in bacterial cells. Moreover, for hepatic function compromised patients, 1-Methyl-3,4-bis(3-indolyl) maleimide, could be used if infected by *N.*

gonorrhoea, A. baumanii, C. coli, K. pneumoniae, E. faecium, H. pylori, P. aeruginosa, S. aureus and *S. typhii.*

These molecules that act upon multiple targets spanning from DNA replication, mRNA transcription, riboswitch modulated suppression of peptide synthesis, or +1 frame shift mutated protein translation to give defunct protein even translated and prevention of lipid biosynthesis for preventing cell membrane biosynthesis could act in multiple targets for pathogen inhibition. These multiple targets would render difficulty to pathogens to easily evolve resistance since these are really critical in its survival and mutating in these targets could be more lethal than survival.

RECOMMENDATIONS

Thus, it is recommended that these molecules be tested *in vitro* for AST and further pursued as potential lead molecule to develop drugs. Respective enzymes inhibition kinetics could be studied and riboswitch structure could also be investigated. The lead molecules could be taken for animal testing and toxicity testing.

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8. APPENDICES

8.1 Data sources for metabolic model reconstruction and refinement

8.1.1 DNA sequence and genome annotation databases

DDBJ	http://www.ddbj.nig.ac.jp/	General nucleotide sequence database
EMBL	http://www.ebi.ac.uk/embl/	General nucleotide sequence database
GenBank	http://www.ncbi.nlm.nih.gov/Genbank/	General nucleotide sequence database
Integr8	http://www.ebi.ac.uk/integr8/	Integrated information on complete genomes
CMR	http://cmr.jcvi.org/	Integrated information on complete prokaryotic genomes
IMG	http://img.jgi.doe.gov/	Integrated system for analysis and annotation of microbial genomes
SEED	http://seed-viewer.theseed.org/	Integrated system for analysis and annotation of genomes using functional subsystems

8.1.2 Protein and enzyme databases

BRENDA	http://www.brenda-enzymes.info/	Comprehensive enzyme information system gathering data collected from the literature by curators
ENZYME	http://www.expasy.ch/enzyme/	Enzyme nomenclature database providing extensive information on all enzymes with an associated EC number
UniProt	http://www.ebi.ac.uk/uniprot/	Universal Protein Resource gathering protein sequences and annotations from SwissProt (manually reviewed), trEMBL (computer annotated), and PIR
TransportDB	http://www.membranetransport.org/	Predictions of membrane transport proteins for fully sequenced genomes
PSORTdb	http://db.psort.org/	Repository of experimentally determined and predicted protein localizations

Prolinks	http://prolinks.mbi.ucla.edu/	Database of predicted functional links between proteins
STRING	http://string.embl.de/	Database of known and predicted protein–protein interactions

8.1.3 Metabolic databases

CheBI	http://www.ebi.ac.uk/chebi/	Database on small molecules of biological interest
Pubchem	http://pubchem.ncbi.nlm.nih.gov/	Database on small molecules
LipidMaps	http://www.lipidmaps.org/	Database on lipid metabolites
Reactome	http://www.reactome.org/	Curated database of biological pathways
KEGG	http://www.genome.jp/kegg/	Suite of databases comprising information on compounds, reactions, pathways, genes/proteins
BioCyc	http://www.biocyc.org/	Collection of organism-specific pathway/genome databases, including a curated multiorganism pathway database: MetaCyc
UniPathway	http://www.grenoble.prabi.fr/obiwarehouse/unipathway/	Curated resource of metabolic pathways linked to UniProt enzyme database
UM-BBD	http://umbbd.msi.umn.edu/	Database on microbial biocatalytic reactions and biodegradation pathways

8.1.4 Experimental data repositories

IntAct	http://www.ebi.ac.uk/intact/	Repository of reported protein interactions
DIP	http://dip.doe-mbi.ucla.edu/	Database of experimentally determined interactions between proteins
Array Express	http://www.ebi.ac.uk/aerep/	Public repository of microarray data
GEO	http://www.ncbi.nlm.nih.gov/geo/	Public repository of microarray data
ASAP	http://asap.ahabs.wisc.edu/	Repository of results of functional genomics experiments for selected bacterial species
E. coli multi-omics DB	http://ecoli.iab.keio.ac.jp/	Comprehensive dataset of transcriptomic, proteomic, metabolomic, and fluxomic experiments for *E. coli* K12

Systomonas	http://www.systomonas.de/	Repository of 'omics' datasets and molecular networks for Pseudomonads species
PubMed	http://www.pubmed.org/	Database on biomedical literature

8.1.5 Metabolic Model Repositories

BiGG	http://bigg.ucsd.edu/	Repository of reconstructed genome-scale metabolic models
BioModels	http://www.ebi.ac.uk/biomodels/	Database of mathematical models of biological systems

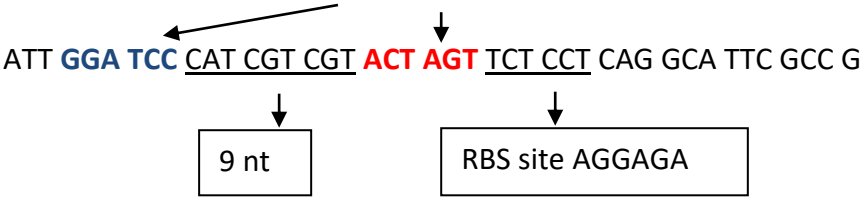
8.2 Software tools for FBA

Constraint-based reconstruction and analysis (COBRA) toolbox	http://www.bioeng.ucsd.edu/research/research_groups/gcrg/downloads/COBRAToolbox/	Interfaces with MATLAB for extensive analysis of networks using FBA; performs gene deletions – single and multiple (can interface with LINDO, GLPK, CPLEX)
MetaFluxNet	http://mbel.kaist.ac.kr/lab/mfn/	Metabolic flux analysis
CellNetAnalyzer	http://www.mpi-magdeburg.mpg.de/projects/cna/cna.html	Structural and functional analysis of cellular networks
SNA: Stoichiometric network analysis	http://www.bioinformatics.org/project/?group_id=546	Mathematical toolbox for stoichiometric network analysis
Yana	http://yana.bioapps.biozentrum.uni-wuerzburg.de/	Network reconstruction, visualization and analysis
PathwayAnalyser	http://sourceforge.net/projects/pathwayanalyser	FBA and MoMA of metabolic networks; gene deletion studies
Systems Biology Research Tool	http://www.bioc.uzh.ch/wagner/software/SBRT/	Multiple methods for analysing stoichiometric networks
SBML Software Guide	http://sbml.org/SBML_Software_Guide	Resource list for software tools, model databases

8.3 Primer design for *cobA* promoter with forward primers 100, 200, and 500bp from the TSS site

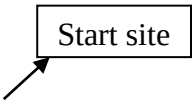
Pseudomonas aeruginosa PA96
CobA gene
Vector puc19

5' end promoter/500 bp upstream TSS with NdeI site (forward)
CTG **CAT ATG** CTA CGG CAA GCG AGT GGC CTG TC
Reverse primer with BamHI and SpeI restriction sites



5' end promoter/ 200 bp upstream with NdeI site (forward)
GCG A**CA TAT G**GC CAA CTT CGG CAG ACT GTG CAG

5' end promoter/ 100 bp upstream with NdeI site (forward)
GTC AAT **CAT ATG** CTC GGC AAG CAA CTG GCG



E.coli lacZ gene 3075 bp
With SpeI site (forward)
GCC G**AC TAG T**AC AGG ATA CAG CT**A TG**A C
With BamHI site (reverse)
ATA C**GG ATC C**TT ATT TTT GAC ACC AGA CCA AC

8.4 Media composition of ISP2 and ISP4

ISP2 growth media

Ingredients	Gms / Litre
Peptic digest of animal tissue	5.000
Yeast extract	3.000
Malt extract	3.000
Dextrose	10.000
Agar	20.000
Final pH (at 25°C)	6.2±0.2

ISP4 growth media

Ingredients	Gms / Litre
Starch, soluble	10.000
Dipotassium phosphate	1.000
Magnesium sulphate. heptahydrate	1.000
Sodium chloride	1.000
Ammonium sulphate	2.000
Calcium carbonate	2.000
Ferrous sulphate, heptahydrate	0.001
Manganous chloride, 7H2O	0.001

Zinc sulphate, 7H2O	0.001
Agar	20.000
Final pH (at 25°C)	7.2±0.2

8.5 Results from OSIRIS property explorer for those passing ADME/Tox filters

Zinc Ids	Total MW	cLogP	cLogS	H-Acceptor	H-Donor	Polar Surface Area	Drugliken ess
ZINC000002504574	209.632	1.7882	-2.855	3	2	53.09	0.39086
ZINC000000407973	209.632	1.7882	-2.855	3	2	53.09	0.39086
ZINC000000393884	202.300	2.2064	-2.037	2	1	19.03	2.9146
ZINC000002382836	236.362	1.9819	-3.458	2	2	85.21	0.7375
ZINC000000174849	202.256	1.5422	-2.411	3	2	44.89	2.846
ZINC000002539813	203.244	0.2213	-2.302	4	3	84.9	0.41041
ZINC000004639456	201.272	1.2245	-2.108	3	2	31.06	2.8408
ZINC000000566973	211.219	2.0177	-3.923	3	1	46	1.0331
ZINC000022001985	216.327	2.5176	-2.445	2	1	19.03	5.7758
ZINC000015120069	216.239	0.6342	-2.682	4	1	59.06	2.5365
ZINC000000066104	218.255	1.1965	-2.115	4	3	65.12	2.8596
ZINC0000	218.255	-1.0554	-2.336	4	3	79.11	0.15853

04240327							
ZINC000001644308	216.283	1.8087	-2.049	3	1	36.1	5.5039
ZINC000012401856	219.239	1.0423	-2.358	4	3	73.32	2.72
ZINC000012401857	219.239	1.0423	-2.358	4	3	73.32	2.72
ZINC000012401854	219.239	1.0423	-2.358	4	3	73.32	2.72
ZINC000012401855	219.239	1.0423	-2.358	4	3	73.32	2.72
ZINC000028003897	232.326	2.1719	-2.149	3	2	39.26	5.704
ZINC000002213438	250.728	2.6026	-3.417	3	2	44.89	2.9986
ZINC000045256608	293.167	1.5819	-3.639	4	3	66.2	0.59036
ZINC000000142608	228.338	2.7242	-2.771	2	1	19.03	5.0085
ZINC000001682922	229.238	0.6903	-2.796	5	3	73.99	4.5285
ZINC000000298332	244.293	0.2348	-2.194	4	2	56.33	2.1366
ZINC000000298330	244.293	0.2348	-2.194	4	2	56.33	2.1366
ZINC000000155234	236.273	2.8465	-3.653	3	2	44.89	0.85682
ZINC000014724327	246.265	-1.3076	-2.018	5	4	85.35	0.51167
ZINC0000	259.332	1.3037	-2.518	4	2	80.22	4.4683

00171154							
ZINC000000171152	259.332	1.3037	-2.518	4	2	80.22	4.4683
ZINC000002018455	244.381	3.4734	-2.907	2	1	19.03	3.5102
ZINC000001996369	244.381	3.2832	-3.123	2	1	19.03	3.1451
ZINC000012371832	282.319	0.5052	-2.736	6	3	107.64	2.4175
ZINC000012371833	282.319	0.5052	-2.736	6	3	107.64	2.4175
ZINC000002539054	260.380	2.9375	-2.827	3	2	39.26	3.089
ZINC000002557704	260.292	1.1747	-2.426	5	3	82.19	1.165
ZINC000004498255	259.308	0.6259	-2.178	5	3	73.99	1.9676
ZINC000005420963	321.261	4.0492	-3.782	2	1	15.27	2.1993
ZINC000002466097	253.348	1.8129	-2.163	3	2	32.75	3.1645
ZINC000045256267	335.204	1.9901	-3.837	5	3	69.28	1.9706
ZINC000000014457	272.347	2.4448	-2.661	4	1	45.33	3.5613
ZINC000049000266	337.260	3.7035	-3.486	3	2	35.5	2.1943
ZINC000011890787	272.303	1.2418	-2.288	5	2	73.4	2.7354
ZINC0000	272.303	1.2418	-2.288	5	2	73.4	2.7354

11890783							
ZINC000049169727	268.319	1.9633	-2.476	5	2	66.74	2.5945
ZINC000049169206	282.346	2.1623	-2.07	5	1	57.95	4.1162
ZINC000002627687	276.338	3.2314	-3.943	3	1	36.1	3.8352
ZINC000001582159	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000001582161	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000001582158	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000001582158	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000001582158	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000001582160	283.330	0.8932	-2.504	5	2	65.2	7.3467
ZINC000000285226	282.317	3.0868	-3.895	3	2	44.89	0.7273
ZINC000000128785	285.346	1.1086	-3.02	5	3	73.99	0.17549
ZINC000000128782	285.346	1.1086	-3.02	5	3	73.99	0.17549
ZINC000004163159	292.337	1.9524	-3.686	4	2	61.96	0.57557
ZINC000004163163	292.337	1.9524	-3.686	4	2	61.96	0.57557
ZINC0000	313.380	1.7413	-3.319	5	2	103.09	5.1599

13375360							
ZINC000028971613	315.396	1.5595	-2.59	5	3	106.25	3.7911
ZINC000053151307	291.397	2.8955	-3.069	3	1	22.27	6.6203
ZINC000049171024	310.356	2.2729	-2.396	6	1	75.02	5.2186
ZINC000049170306	310.400	3.0323	-2.499	5	1	57.95	2.0487
ZINC000003230765	344.825	2.8166	-2.855	5	2	90.43	4.265
ZINC000014824027	306.364	2.9695	-3.655	4	3	65.12	0.83676
ZINC000000968255	336.430	1.7048	-3.534	6	3	87.66	5.5213
ZINC000000156792	336.430	1.7048	-3.534	6	3	87.66	5.5213
ZINC000049169056	316.407	3.3464	-3.069	4	2	47.71	3.7658
ZINC000005600522	336.394	1.0155	-3.32	5	2	90.27	4.151
ZINC000101578473	335.386	1.5763	-3.738	5	2	99.62	5.4692
ZINC000096113202	322.407	2.7262	-3.05	4	1	37.49	1.6354
ZINC000225492550	336.438	1.7093	-3.031	5	2	55.39	1.8988
ZINC000006096559	333.390	1.8772	-3.615	5	3	73.99	4.4232
ZINC0000	333.390	1.8772	-3.615	5	3	73.99	4.4232

02560888							
ZINC000004899716	333.390	1.8772	-3.615	5	3	73.99	4.4232
ZINC000006096622	333.390	1.8772	-3.615	5	3	73.99	4.4232
ZINC000049171033	336.394	2.591	-2.912	6	1	75.02	6.3735
ZINC000002560262	327.342	1.5839	-3.555	5	3	77.75	3.3968
ZINC000013369641	356.449	1.5699	-2.864	6	3	115.12	4.9882
ZINC000013677763	336.390	2.8995	-3.673	5	3	74.35	0.92273
ZINC000058535134	352.393	0.1634	-2.921	6	3	110.5	4.2533
ZINC000004520331	349.433	2.4271	-3.691	5	3	73.99	0.26291
ZINC000004520329	349.433	2.4271	-3.691	5	3	73.99	0.26291
ZINC000019909549	344.461	2.8578	-3.072	4	2	38.06	6.9425
ZINC000002560263	341.369	1.8368	-3.193	5	2	68.96	3.8631
ZINC000225494147	350.464	2.1072	-3.399	5	2	55.39	1.8354
ZINC000072107870	357.409	1.1208	-2.76	7	2	89.95	6.1701
ZINC000033836374	353.464	2.5432	-3.839	5	2	61.44	0.019812
ZINC0000	347.461	3.4871	-3.768	4	2	48.13	8.831

00001567							
ZINC000004899565	349.389	1.5315	-3.319	6	4	94.22	4.4198
ZINC000014860718	365.432	0.7214	-3.44	6	2	78.51	5.1225
ZINC000003780014	374.439	0.8241	-3.246	8	5	123.32	3.5518
ZINC000015219763	358.400	1.8459	-3.702	6	2	77.56	6.1517
ZINC000015219760	358.400	1.8459	-3.702	6	2	77.56	6.1517
ZINC000049171027	372.427	3.7167	-3.566	6	1	75.02	5.3617
ZINC000001494627	379.459	1.9496	-3.541	6	4	88.59	2.4488
ZINC000040860915	383.447	1.2707	-3.314	7	3	98.74	2.2205
ZINC000072110006	399.446	1.6054	-3.17	8	1	96.02	6.0494
ZINC000014687876	389.458	1.744	-3.848	7	3	90.12	0.93977
ZINC000038796449	395.458	1.2882	-3.11	7	1	78.95	6.4971
ZINC000045495541	393.442	1.3651	-3.212	7	1	78.95	6.9528
ZINC000058537879	409.445	-2.144	-2.688	8	4	139.6	1.5312
ZINC000096923254	418.452	0.95	-3.631	8	3	111.1	5.4268
ZINC0000	445.478	0.6841	-3.507	9	3	114.34	6.9585

15219741							
ZINC000040420740	443.462	0.7152	-3.671	9	2	103.34	6.8191
ZINC000040393229	465.548	2.4241	-3.831	8	3	102.34	0.25201

8.6 List of target proteins with sources

Proteins	Pdb Ids	Ligand	Organisms	References
metK (S-adenosyl methionine synthase)	5T8S	SAM	Neisseria gonorrhoea	Dranow *et.al,* To be published
cysG (Siroheme synthase)	1PJQ	S-adenosyl-L-homocysteine (SAH)	Salmonella typhimurium (strain LT2/ATCC 700720)	Stroupe *et.al*
cobA (Uroporphyrin-III C-methyl transferase)	1V9A	SAH	Thermus thermophilus ATCC 27634	Rehse *et.al*
dam (DNA adenine methyltransferase)	2DPM	SAM	Streptococcus pneumoniae	Tran *et.al*
trmD(tRNA (Guanine-N(1)-)-methyltransferase)	4YVG	SAM	Haemophilus influenza	Ito *et.al*
SAM-I riboswitch	4KQY	SAM	Bacillus subtilis	Lu *et.al*

8.7 Indole derivatives with higher binding potential in metK than SAM

Energy minimized ligands	B.E in metK (B.E of native ligand SAM=-8.7)
ZINC000000128782_uff_E=1347.97	-9.1
ZINC000000128785_uff_E=1378.65	-9.9
ZINC000000298330_uff_E=382.97	-9.6

ZINC000000298332_uff_E=355.22	-9.5
ZINC000004520329_uff_E=369.53	-10.4
ZINC000004520331_uff_E=366.48	-10.6
ZINC000058535134_uff_E=596.69	-10.3
ZINC00014457_uff_E=374.30	-9.4
ZINC000225492550_uff_E=463.82	-9.5
ZINC00285226_uff_E=393.14	-10.2
ZINC00566973_uff_E=502.92	-9.1
ZINC01494627_uff_E=503.10	-9.3
ZINC01682922_uff_E=471.08	-9.9
ZINC02466097_uff_E=536.70	-9
ZINC02539813_uff_E=340.00	-9.1
ZINC02557704_uff_E=336.02	-8.9
ZINC02560262_uff_E=1016.24	-10.7
ZINC02560263_uff_E=1033.97	-10.1
ZINC02627687_uff_E=586.11	-9.9
ZINC03230765_uff_E=913.72	-9.5
ZINC03780014_uff_E=437.82	-8.9
ZINC04163159_uff_E=399.16	-9.9
ZINC04163163_uff_E=399.35	-10.1
ZINC04240327_uff_E=347.88	-9
ZINC04498255_uff_E=341.97	-9.3
ZINC04639456_uff_E=451.82	-9
ZINC04899565_uff_E=450.84	-10.4

ZINC04899716_uff_E=446.34	-10.2
ZINC05600522_uff_E=713.54	-9.4
ZINC06096559_uff_E=448.34	-10.4
ZINC06096622_uff_E=523.73	-10.8
ZINC11890783_uff_E=475.43	-9.2
ZINC11890787_uff_E=478.60	-9.3
ZINC12371832_uff_E=798.65	-9.1
ZINC12371833_uff_E=810.05	-9.2
ZINC12401854_uff_E=333.01	-9.2
ZINC12401855_uff_E=331.69	-9.5
ZINC12401856_uff_E=331.13	-9.2
ZINC12401857_uff_E=333.09	-9.3
ZINC13369641_uff_E=592.78	-9.6
ZINC13375360_uff_E=571.04	-10.2
ZINC13677763_uff_E=415.93	-9.7
ZINC14724327_uff_E=388.92	-9.2
ZINC14824027_uff_E=389.74	-9.9
ZINC15120069_uff_E=149.70	-9
ZINC15219763_uff_E=554.59	-9.5
ZINC19909549_uff_E=694.06	-10.6
ZINC28971613_uff_E=591.01	-10.3
ZINC45256267_uff_E=450.75	-11.7
ZINC45256608_uff_E=389.26	-10.8
ZINC49169056_uff_E=836.61	-10

ZINC49169206_uff_E=785.17	-10.5
ZINC49169727_uff_E=717.99	-11.1
ZINC49170306_uff_E=794.00	-10.6
ZINC49171024_uff_E=718.10	-10.8
ZINC49171033_uff_E=1989.71	-10
ZINC53151307_uff_E=504.32	-10.5
ZINC96113202_uff_E=575.72	-10.1

8.8 Docking results of six lead indole derivatives in human homologue of MetK, Mat2A (RCSB id: 4KTT)

Ligands	Binding Energy
ZINC02560263_uff_E=1033.97	-7.3
ZINC04899565_uff_E=450.84	-10.8
ZINC06096622_uff_E=523.73	-10.2
ZINC15219763_uff_E=554.59	-9.8
ZINC49169056_uff_E=836.61	-10.3
ZINC49171024_uff_E=718.10	-10.2
SAM (native ligand)	-8.7

8.9 Docking results of six lead indole derivatives along with a known GSK inhibitor in Glycogen Synthase Kinase 3 (GSK 3)

Ligands	Binding Energy
ZINC02560263_uff_E=1033.97	-8.1
ZINC04899565_uff_E=450.84	-9
ZINC06096622_uff_E=523.73	-8.9
ZINC15219763_uff_E=554.59	-9.4
ZINC49169056_uff_E=836.61	-9.1

ZINC49171024_uff_E=718.10	-8.8
N-(4-Methoxybenzoyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea	-7.6

8.10 Protein preparation using AutoDockTools (ADT) and ligand preparation using OpenBabel

The pdb file format of target protein from RCSB or the ones computationally derived through homology modeling were loaded in ADT where, water molecule was removed first under 'edit' option, add hydrogen, merge non-polar and finally compute gasteiger under 'edit' option and 'charges' subheading.

The ligands were downloaded on mol2 file formats for those with unavailable pdbqt file formats. All these were energy minimized using OpenBabel in PyRx software to increase molecular flexibility prior to docking.