UNIVERSITY OF
BIRMINGHAM

Project 1 *Molecular genetic analysis of m6A methylation of mRNA in a Drosophila Model*

And

Project 2 *Development of a phenotypic highthroughput-screening assay to identify novel molecules that inhibit Mycobacteria.*

By

Miss Panchali Kanvatirth

A combined research thesis submitted to the University of Birmingham as part of the requirement for the degree of Master Of Research in Molecular and Cellular Biology.

College of Life and Environmental Sciences

School of Biosciences

University of Birmingham

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Dear Reader,

This thesis is a combined dissertation of two individual projects undertaken as a compulsory part of a research masters degree in molecular and cellular biology.

My first project was under the supervision of Dr. Matthias Soller involving the study of a specific type of methylation using *Drosophila* as a model organism. It involved developing a knockout mutant of a gene CG6422 that encodes a YTH domain containing protein which might interact and control neuronal development. The project was conducted at the School of Biosciences, College of Life and Environmental Sciences, University of Birmingham.

Dr. Luke Alderwick supervised my second project at the institute of microbiology and infection at the School of Biosciences, College of Life and Environmental Sciences, University of Birmingham. The project involved use of automated liquid handling system and highthroughput screening to design and develop a screening assay to screen novel antimycobacterial compounds and drugs.

Yours faithfully,

Panchali Kanvatirth

Molecular genetic analysis of m6A methylation of mRNA in a Drosophila Model

By

Panchali Kanvatirth

Project 1 - MRes in Molecular and Cellular Biology

Supervisor: Dr. Matthias Soller

School of Biosciences

University of Birmingham

March 2013

Acknowledgment

Foremost I offer my sincerest gratitude to my supervisor, Dr. Matthias Soller, who has supported me throughout my thesis with his patience and knowledge and giving me an opportunity to learn about this field of research. I would like to thank Dr. Irmgard Haussmann, for all her help and sound advice during the period of study at the lab.

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List Of Abbreviations

ADAR - Adenosine Deaminase Acting on RNA

bp - Base pair

CPSF - Cleavage and Polyadenylation Specificity Factor

CTD - Carboxy-Terminal Domain / Carboxyl Tail Domain

DNA - Deoxyribonucleic Acid

DSE – Downstream Sequence Element

dNTPs - Deoxy nucleotide Triphosphates

ECS – Editing site Complementary Sequence

EDTA - Ethylenediaminetetraacetic Acid

ELAV - Embryonic Lethal Abnormal Vision

GMP – Guanosine monophosphate

g - Gram

h - Hour

min - Minute

mL - Millilitre

^{o}C - Degree celsius

PBS - Phosphate Buffer Saline

PBT - Phosphate Buffer saline with Triton- X/ Tween 80

PCR - Polymerase Chain Reaction

RNA - Ribonucleic Acid

RNP - Ribonucleoprotein

RRM - RNA Recognition Motif

s - Second

TAE - Tris Acetate EDTA

μL - Microlitre

Abstract

Living systems are governed by tightly regulated systems and pathways, which determine the survival time, development and period of division of the cell or an organism. All genetic information regarding the body is encoded in the DNA of an organism, which is processed into functional forms such as RNA or proteins. Several pathways involving processing of RNA regulate the synthesis of RNA and protein. Among these processes internal methylation of adenosine bases is seen to regulate alternate splicing and regulate neuronal development. The proteins binding to these methylated sites on the RNA are seen to have a special domain called an YTH domain. This study involved developing a knockout mutant of one of such proteins containing this domain, which might interact and control neuronal development called CG6422. A single knock-out mutant was obtained for the said gene and further phenotypic and behavior studies were done to observe a level of neuronal defect. Further studies and mapping of the knock-out gene could reveal the exact function and interaction of the protein with methylated RNA.

TABLE OF CONTENTS

1. INTRODUCTION

A genome contains hereditary information in the form of nucleic acid molecules. These molecules have discrete functional units; genes, which are the basis to all the characteristics that an organism has. Living organisms have genes varying in number such as < 500 (for mycoplasma), >20,000 in humans to around 60,000 in a protozoan species (Lewin *et. al.*, 2004).

The next most important nucleic acid apart from the DNA is the RNA, which plays a central role in gene expression. It acts as a central player in converting genetic information from the DNA or mainly genes into active proteins, which essentially make the organisms work (Evan, 1991).

There is compartmentalization of the process of conversion of DNA to RNA (transcription) and RNA to protein (translation) in eukaryotes by a nuclear membrane. The RNA is transported after processing through the nuclear pore into the cytoplasm so that translation can occur (Lewin *et. al.*, 2004; Evan, 1991).

Eukaryotic cells have three types of genetic controls: transcriptional, post-transcriptional and translational.

- The transcriptional control involves the regulation of pre-mRNA synthesis.
- Post-transcriptional modification involves mRNA modification after its synthesis in the nucleus, which is important for the regulation of gene expression.
- Translational control involves control of intracellular protein synthesis

(Soller, 2006; Orphanides *et. al.*, 2002)

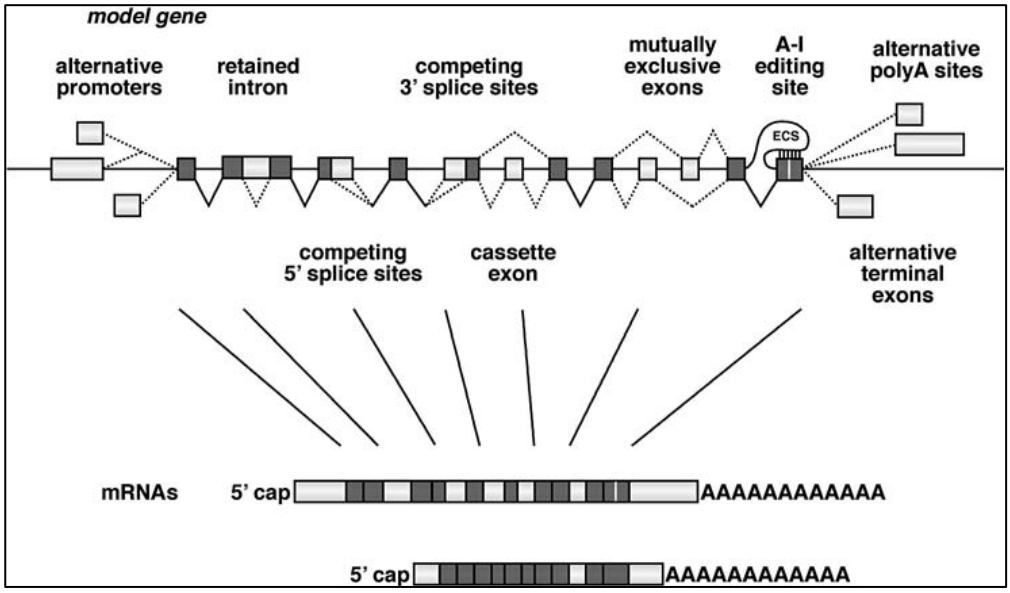


Figure 1. The exons in the figure are depicted as boxes, introns as lines. Alternative mRNA processing types are given: The gene on top and mRNA isoforms below. Alternative mRNA processing is depicted by dotted lines while constitutive ones with solid lines. Adenosine to inosine editing (A to I), have editing site complementary sequences (ECS) present in the introns with edited site in the exon (Soller, 2006).

1.1Post -transcriptional regulation

The RNA before it is processed is known as a pre-mRNA and undergoes various modifications before it forms an mRNA, which is transcribed into proteins. These modifications are also termed as post-transcriptional modifications or RNA processing. The RNA processing involves modifications such as capping, polyadenylation, methylation, editing and splicing as shown in Fig.1. Some of them are essential to the formation of mRNA and its function such as capping,

polyadenylation and splicing while others are regulated based on their requirement (Soller, 2006).

There is evidence supporting coupled occurrence of pre-mRNA splicing and 3' polyadenylation with 5' capping processes. It has been biochemically proved that an intact 5' cap is required for efficient splicing and polyadenylation (Orphanides *et. al.*, 2002). The major player here is the CTD tail of the mRNA, which is phosphorylated and dephosphorylated at various stages of transcription and also changes during other processing events such as polyadenylation, splicing and capping. (Cowling, 2010).

The RNA processing steps will be discussed further in sequence:

1.1.1 Capping:

Capping of mRNA involves addition of a 7-methyl guanosine "cap" to the first transcribed nucleotide. In yeast and humans it has been seen to be important for efficient gene expression and viability of the cell. The 7-methyl cap is required for translation as it stabilizes the RNA against attachment of exonucleases that destroy it. It also helps in promoting transcription and splicing, polyadenylation and nuclear export of mRNA. RNA polymerase II is the main enzyme involved which interacts with the cap methyltransferase (Cowling, 2010).

The steps of capping involve a three -step reaction where in a N7 methyl GMP is added onto a 5'-5' triphosphate linkage which is then again bound by a cap binding protein complex of 20-80 kDa (Soller, 2006)

1.1.2. Polyadenylation:

Polyadenylation occurs at the 3' end of the pre-mRNA where the consensus sequence of AAUAAA hexamer is bound by a cleavage and polyadenylation specificity factor (CPSF) and a cleavage stimulatory factor (CstF) binds to a U- or GU-rich downstream sequence element (DSE). After cleavage under direction of two additional proteins, cleavage factors II and I and poly A polymerase, a tail of about 200 adenosine residues is added (Soller, 2006).

1.1.3. RNA editing:

RNA editing is a process of creating variation by changing nucleotides in the RNA and seems to occur only in eukaryotic cells and in bacteria among prokaryotes. The process occurs mainly through deamination of residues, which code for different amino acids therefore creating variation. The most common conversion is that of adenosine to inosine which is carried out by the enzyme adenosine deaminase acting on RNA (ADAR). Another less common editing occurs for cytidine to uridine demamination, which is found only in two genes to date. The fact that adenosine deaminase acting on RNA-2 (ADAR2) is self-regulatory and changes the splicing pattern and that most ECS (editing site complementary sequence) are present in introns, it is obvious that RNA editing occurs before splicing and affects it to a certain extent (Soller, 2006).

1.1.4. Splicing:

Splicing involves removal of introns and joining the exons together into a functional mRNA ready to be transcribed into a protein in the cytoplasm. It occurs in two trans-esterification reactions leading to a spliced mRNA while removing introns in a lariat loop formation.

The whole splicing assembly involves consecutive addition of five small ribonucleoproteins (Snurps) which are U1, U2, U4 and U6 and other proteins to form a complete complex called the spliceosome. On the pre-mRNA a 5' splice site with a consensus sequence, a branch point, a polypyrimidine tract and a 3' splice site are required to splice the pre-mRNA.

Another type of splicing occurs where in splicing out different introns. It can lead to variable combinations and diversity in the same RNA sequence therefore, producing variety in the proteins that are thus transcribed from these mRNAs. This is known as alternative splicing. This involves the presence of a large number of RNA binding proteins (RBPs) containing one or more RNA binding domains and at least one additional auxiliary domain. In Drosophila, gene specific RNA binding protein ELAV is known to bind to the *ewg* gene. The protein is said to have evolutionary conserved domains, which is involved in neuronal regulation (Soller, 2006).

1.1.5. RNA methylation:

Capping at 5' end involves methylation of the initial bases, but many mRNAs undergo internal methylation. The major form of internal methylated base is N6

methyladenosine, found in eukaryotic mRNA, viral mRNA and retroviral RNAs. Although the methylation is sequence specific for Gm6AC and Am6AC, the distribution of the methylated bases is random (Kane *et. al.*, 1985).

The only modification in mRNA capping that is well studied is the m7G having a role in translation initiation, mRNA transport, splicing and degradation of mRNA.

The Eukaryotic mRNA undergoes various types of methylation such as :

- The standard methylation, which occurs during 5' capping of the mRNA strand. It involves the formation of M7G (5') ppp (5') N1 (m) pN2 (m) pNpNpN… - N7 methylguanosine (m7G) 5' cap structure at the 5' end (Fig.2) protecting the mRNA from degradation. The length of the cap has been known to determine the time and importance of the protein encoded by the mRNA (Jia *et. al.*, 2013).

- The next type of methylation is N6-methyl-2'-O-methyladenosine (m6Am). Here, the first position of 5' termini (N1) and the internal 6^{th} position (N6) are methylated. It defines the difference between self and non-self mRNA and is said to be the molecular signature of the host (Jia *et. al.*, 2013) (Fig.2).

- 2'-O- methylated nucleoside (Nm) – the first two starting positions at the 5' terminus (N1 and N2) (Jia *et. al.*, 2013) (Fig.2)

- Internal methylation-
  - 5-methylcytosine (m5C)
  - N6- methyladenosine (m6A) (Jia *et. al.*, 2013) (Fig.2)

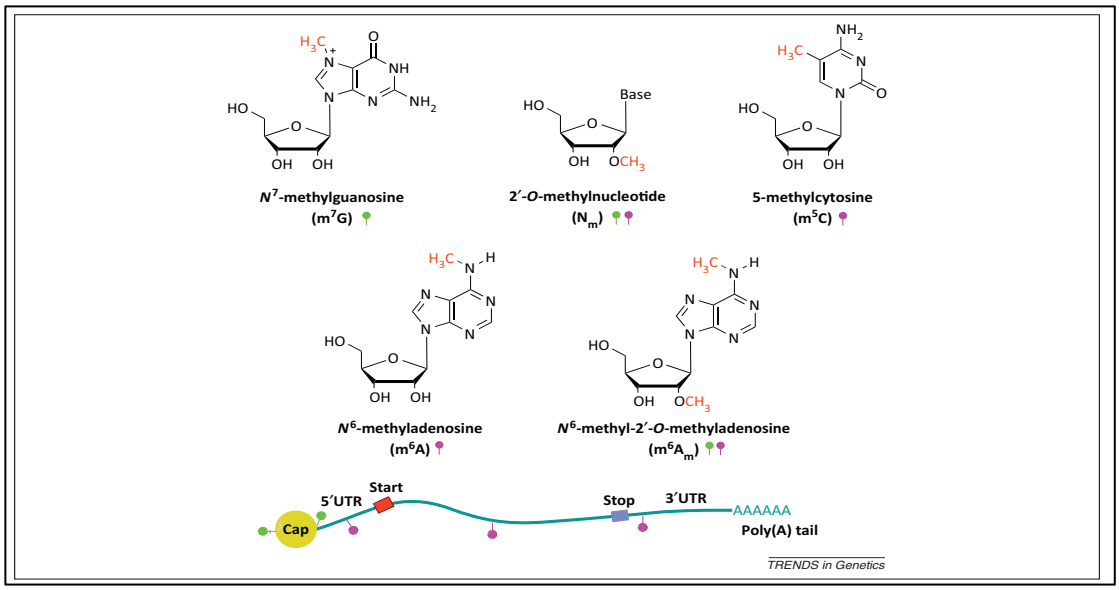


Figure 2. The structure of various types of methylation in RNA is shown. The green ball indicates methylation in the cap and the pink ones indicate internal methylation. (Jia *et. al.*, 2013)

Methylation has been found in eukaryotic cell mRNA and viral mRNAs as well. An enzyme N6 methylase that methylates mostly internal adenosine sites performs internal methylation of pre-mRNA. On an average 1-3 adenosine residues are methylated in an mRNA and they occur both in introns and exons, which proves that the process occurs before splicing. The function of this type of methylation is not clearly indicated though it has been seen to have a role in nuclear processing of mRNA. The selection of the internal adenosine residues is partially governed by the recognition of consensus sequences such as G<u>A</u>C or A<u>A</u>C (the underlined residue being methylated). Although, only a subset of these sequence get methylated additional sites and sequence constraints play a role in site recognition. Recognition of specific sites for m6A methylation has only been found in bovine prolactin and Rous sarcoma virus (Rottman *et. al.*, 1994)

The most prevalent internal methylation is the m6A methylation and its role is still quite unclear. This type of methylation was first found in mammalian mRNA in the mid-1970s. It was found in eukaryotes mainly animals, plants, flies, yeast in its meiotic state and viruses (mainly the Rous sarcoma virus).

The m6A methylation is done by N6 methyltransferase which is a complex of 200kDa having a 70kDa SAM binding subunit structure called MTA70. The remaining structure has not been characterized. The identification of internally methylated sites was difficult early, as the internal methylation does not alter its base pairing property. Therefore the internal m6A could not be mapped using RT base methods (Bokar *et. al.*, 1997).

The m6A sites are highly conserved in humans and mouse. In yeast though methylation is only detected during meiosis and not in the mitotic phases. A study showed that when yeast enters meiosis m6A methylation increases. Also, they indicated that it decreased when the yeast was growing in a low nutrition medium as opposed to a high one and increased when the nutrition was increased. Therefore, the internal methylation controls the fate of meiosis in yeast cells.

M6A methylation was found in monocot plants such as maize, wheat, and oats around 30 years ago. It has been widely studied in Arabidopsis where it is around 1.5% in ratio (m6A to A) (Bodi *et. al.*, 2010; Jia *et. al.*, 2013).

m6A modification in Drosophila was first discovered in 1978. A study on the MT-A70 homolog in drosophila Dm ime4 showed expression in ovaries, which localize in germ line cells in ovaries and somatic cells as well. Complete deletion of Dm ime4 proved lethal for the organism and therefore showed that the gene was essential for

organism viability (Jia *et. al.*, 2013).

As mentioned above the internal methylation on adenosine was difficult to detect which has stunted the study of this type of methylation and its function for a long time. A method for detection of methylation by immunoprecipitation has been deduced using anti- m6A anitbodies against digested RNA transcripts. After which the precipitated molecules can be sent in for sequencing. Domissini *et. al.*, (2012) have deciphered a consensus sequence, which is found in methylated RNA, shown in the fig.3 along with the above said method. This methylation is said to have some regulatory roles due its variable presence in various RNA transcripts.

The consensus sequences were found in higher concentration at the transcription start site (TSS) and the 5' UTR and stop codon. It was also observed that m6a methylation was increased in genes that were moderately expressed as compared to genes that are very highly expressed or have very low expression. m6A methylation has a role in splicing as well, as it is correlated with methylation of multi isoform genes and alternate splicing (Fig.3).

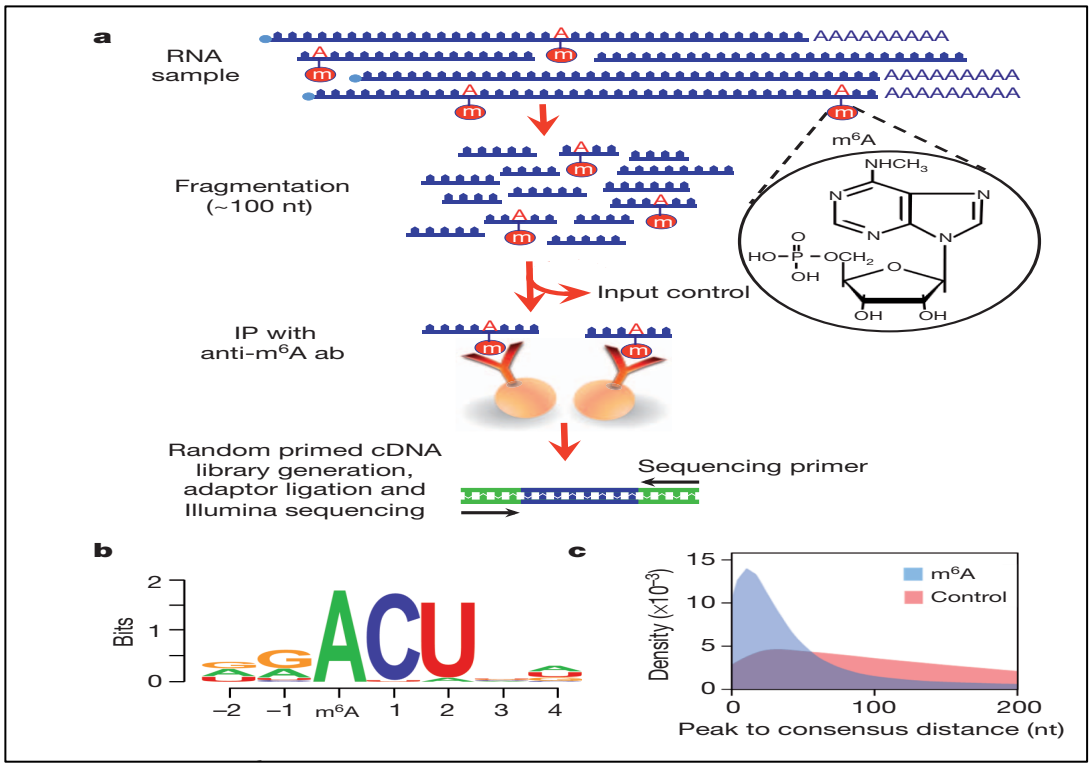


Figure 3. m6A – seq capture of modified RNA fragments exposes an enriched motif. a. Diagram representing the method used to detect m6A methylation using anti-m6A Ab and immunoprecipitation.b. Consensus motif representing by the sequence logo.c.Density plot describing the distribution of distance between the peaks of m6A/control fragments and the nearest m6A consensus motif (RRACU) (Dominissini *et. al.*, 2012)

Meyer *et. al.*, (2012) confirmed the presence of m6A methylation being higher at the 3' UTR and stop codon region as compared to the 5' UTR. The importance of m6a methylation in regulatory roles was confirmed by the demethylase activity of the human FTO (Fat mass and obesity associated) gene, which is responsible for cause metabolic diseases and increasing obesity in humans. The occurrence of m6A methylation in 3'UTR is significant as the region is valued for its role in RNA

stability, subcellular localization, and translation regulation. These processes involve RNA binding proteins, which bind to consensus sequences and other elements in the 3'UTR. The presence of methylated nucleotides can affect regulation of mRNA by modulating the ability of RNA binding proteins to the RNA.

1.2 RNA binding proteins

Recent analysis on m6A RNA for identification of novel m6A binding proteins revealed interaction of proteins with YTH domains and ELAV proteins that bind to the methylated base (Dominissini *et. al.*, 2012). Proteins with YTH domains are conserved in most eukaryotes and bind to RNA functioning mostly in regulating gene expression (Zhang *et. al.*, 2010).

In humans, genes HuB, HuC, HuD and HuR belong to the family of neuron-specific genes called the nELAV gene. These were detected as targets for autoantibodies in patients with paraneoplastic neurological disorders. When the nELAV genes were cloned, sequenced and run against the sequence database, orthologous genes in *Drosophila melanogaster* were found. These genes were the embryonic lethal abnormal visual system genes, which code for neuron-specific nuclear RNA binding proteins (RBP), which regulate alternative splicing in mRNAs. Deletion of these genes leads to embryonic lethality and defects in the nervous system imparing the neuropil therefore symbolic of its name (Pacsale *et. al.*, 2007). The ELAV protein family in Drosophila has three proteins ELAV, Rbp9 and Fne (Toba and White, 2008). An ortholog of the gene has been recognized in *C. elegans*, while a

corresponding family is also present in the vertebrates, zebrafish and *Xenopus* (Pascale *et. al.*, 2007). Splicing efficiency of protein acting on specific splice sites and their stability is affected by ELAV. One of the important roles of ELAV is generation of isoforms of proteins in neurons and ensuring that they are correctly spliced. It is seen to interact with three main genes *ewg*, *nrg* and *arm*. While its function is best understood in relation to the *ewg* gene, the others are thought to have some role in neural development (Koushika *et. al.*, 2000).

The major target of the ELAV proteins is the transcription factor gene *ewg*, which restricts the synaptic growth at neuromuscular junctions (NMJs) (Huassaman *et. al.*, 2008). ELAV proteins have a characteristics structure with three RRMs wherein the second and third RRMs are separated by a "hinge" region of 60-80 amino acids (Toba and White, 2008).. The three RRMs are highly conserved wherein RRM3 is less conserved compared to the other two (Soller *et. al.*, 2010). The ELAV protein interacts with RNA by multimerization and that RRM3 is important for the protein to form a complex multimer. Also RRM3 seems to interact with both proteins as well as RNA through a bi-functional domain (Toba and White, 2008). The RNA binding part of the ELAV is conserved in 12 species of Drosophila with very few amino acid changes (Soller *et. al.*, 2010). The RRM domains have two important consensus sequences RNP1 and RNP2 for binding to RNA (Zhang, 2010). The RNP (Ribonucleoprotein) are embedded in a larger protein domain of 80-100 amino acids. The ELAV proteins have three such RNA binding domains, containing an amino terminal rich in alanine and glutamine (Yao and White, 1999). Both these proteins have consensus sequences, and an octapeptide sequence for RNP1 and a hexapeptide sequence for RNP2 but the RNP2 sequence is less conserved compared to RNP1

(Robinow *et. al.*, 1988). In the ELAV protein binding to the *ewg* gene, the 3' site shows higher affinity to binding and helps in complex formation. Splicing in neurons and binding capacity is regulated by presence of a U rich region in the *ewg*. It seems that unique sequences do not deduce the binding sites, but they provide prospect for combinatorial binding and multiple binding modes of ELAV proteins to the RNA as well as other RNA binding proteins (Soller *et. al.*, 2010).

In recent times, Zhang *et. al.*, 2010, observed a highly conserved domain for RNA binding. It was identified by comparing it with the splicing factor YT521-B therefore the domain was named YTH. The YTH domain is located at the center of the protein and has properties similar to RRM and has RNA binding capacity (Stoilov *et. al.*, 2002). The domain has amino acids from 100 to 150 and is characterized by 14 invariant and 19 highly conserved residues. The proteins from this family that are only found in vertebrates have roles in alternate splicing and molecular regulation. Splicing requires three essential conserved residues in the YTH domain for selecting the correct splice sites. There are 14 residues seen conserved throughout the evolutionary tree which when tested are responsible for detection and selection of splice sites. It was seen when the residues of this region were mutated the proteins were unable to regulate splice site selection. The multimerization and complex formation is facilitated by the ability of the YT521-B carboxyl terminal binding to each other. (Zhang *et. al.*, 2010). The YTH domain proteins bind to m6A-methylated sites on RNA therefore influencing regulation and splicing of pre-mRNA molecules. (Dominissini *et. al.*, 2012).

1.3 Role of m6A methylation in translation

Formation of a peptide sequence from an mRNA utilizing ribosomal machinery is defined as translation. The m6A methylation is to quiet an extent responsible for mRNA splicing, processing and transport as it occurs in the 3' UTR region. Therefore it regulates the production of particular proteins to an extent, which in turn affects the translation process. (Carmody and Wente, 2009) although this functional effect is still not proven and can be detected if the methylation does not occur of if regulatory proteins such as ELAV proteins or proteins with an YTH domain are not produced to help in regulation. This would probably lead to a level of phenotypic features, which can be analyzed.

2. RATIONALE OF STUDY

The proteins involved in m6A methylation and the process itself are conserved within eukaryotes. Therefore to study various effects of these proteins on the organism development we need to utilize a stable model organism comparable to the human system. Biological pathways of humans and fly genomes show a high degree of conservation (Bilen *et. al.*, 2006). Drosophila melanogaster has been studied as a model organism for more than 100 years now, and is still considered the best organism for comparing human biology. It is the best model for comparing the molecular and cell biology, genetics and even behavioral patterns such as circadian rhythms, geotaxis etc. which are similar to humans. For human diseases proteins can be manipulated to see the effect on the fly, which has worked for many human neurodegenerative diseases (Beckingham *et. al.*, 2005).

As explained above, the role of ELAV proteins and proteins with YTH domains are still quiet unclear. In order to study their roles in neural development the genes producing YTH domain containing proteins that are thought to interact with m6A methylated RNA was knocked out. DNA contains mobile elements, which move around in the genome causing disruption of genes, called transposons. They are present in eukaryotes and their family consists of more than hundreds of such genes. They tend to replicate and increase in number within the genome. (Lynch and Conery, 2003). The main aim of this experiment is to allow disruption of the CG6422 gene via transposons. This should result in reduction or alteration in regulatory function of mRNAs in neural cells. The lab already has knockout mutants for a subunit of the methylase gene, which has certain phenotypic features. The knock out mutants for

YTH domain containing proteins from the gene CG6422 in drosophila should also give certain phenotypic features, which is compared to the dMTA70 mutant line (Clancy *et. al.*, 2002; Meyer *et. al.*, 2012; Robida *et. al.,*). CG6422 belongs to the YT521-B like family which affect splicing by selection of splice sites in vivo (CG6422).

Epitope tagged transgenes for the dMTA70 have been created which when stained with fluorescent antibody for epitope-tags and ELAV may detect development of neurons in brains during larval stages (Koushika *et. al.*, 2000).

3. AIM AND OBJECTIVES

3.1 Aim: Molecular Genetic analysis of m6A methylation of mRNA in a Drosophila model

3.2 Objectives:

1. Generate knockouts of CG6422 (96B17) by imprecise excision of nearby transposable elements.

2. Analyse phenotype of these mutants and compare with phenotypes of other flies (Wild type and ELAV mutants dMT-A70).

3. Analysis of dMT- A70 expression in 3' epitope tagged transgenes in larval brains.

4. MATERIALS AND METHODS

3.1 Fly Stocks And Maintenance

Fly stocks and the experiments were maintained and conducted at 25°C on a 12:12 hr. light: dark cycle at a constant humidity using a standard sugar-yeast medium.

3.1.1 Preparation Of Fly Food

Requirements: For 1.75 litres of food : Water- 1700 mL,Agar- 18.0 g,Nipagin- 50 mL,Dextrose- 150g, Cornmeal- 170g, Yeast- 30g (+ 20 %) - 36g

Procedure: The mixture was boiled till the agar is dissolved and homogenously mixed with the other components and dispensed in clean vials at a volume of 10mL. The food was then allowed to cool and solidify following, which it was plugged using sterile cotton plugs. The food was then stored at 4°C until use.

3.1.2 Fly Stocks (From Bloomingtion):

- EP 21985: w118, P {EPg} HP 35572

Transposable element insertion site; P {EPg} – inserted element; similar to P {EP} but modified to work in female germ line; Mobile activating element 8.057Kb

- CxD TM3Sb; w118; TM3, Sb1/CxD

White eyed flies with chromosome 3 effectively balanced and has the stubble hair marker, crossing over strongly reduced in chromosome 3 except distal half of 3L

- 24998 TM3Sb Df; w118; Df (3R) BSC 494/TM6C, Sb1, Cu1

Df (3R) BSC 494- chromosomal deletion, has a stubble marker

- T36 – White eyed flies with two balancer markers, for stubble bristles and tubby larva

3.1.3 Identifying Flies:

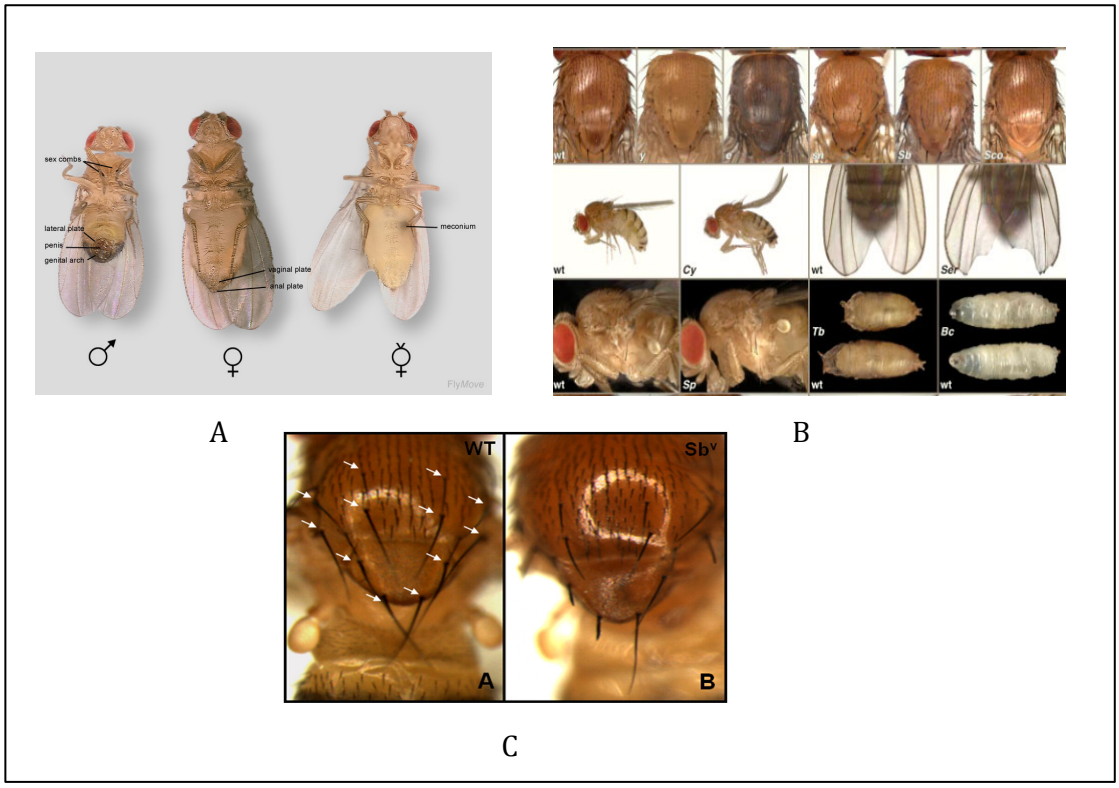


Figure 4. A. Sex differentiation with male flies having sex combs, penis and a genital arch while females have a vaginal plate and virgins have a meconium spot on the abdomen. B. Multitude of phenotypes from body colour, bristle type, wing type and shape, larval size. C. Difference between a wild type bristle and stubble bristles. (Song *et. al.*, 2007)

Phenotypic identification of flies was done using comparative study referring pictures and microscopic study of flies.

Male and female sexing of flies is done on basis of presence of sex combs on the fore legs of males and identifying a black patch at the dorsal hind region of the fly. Also generally male flies are smaller than female flies.

The crossing scheme involved getting female virgins to set up crosses which were identified by the presence of a meconium as shown in the figure above. Female virgins generally are lighter in comparison to older flies and can be collected at regular timings, as they do not sexually mature within 8-12 hours of emergence.

There were other phenotypic features, which were to be identified such as stubble hair, tubby markers. The stubble hair marker was identified by the presence of short, thick bristles on the thorax as opposed to long tapered bristles seen in wild type flies as in the above-mentioned figure.

The tubby markers are hard to identify in adults but can be easily detected in the larval or pupal stages, as they are much smaller and fat compared to non-tubby ones. In adult flies these are differentiated using the presence of dark patches on the thorax of the flies.

3.2 PCR Screening

3.2.1 Primer Designing

Primer pairs were designed to aid screening of the knock out mutant flies.

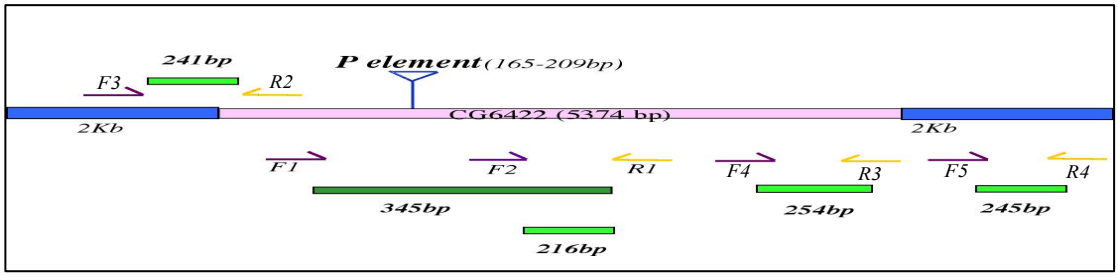


Figure 5. The primer pair positions are depicted in the diagram with sizes of amplified products.

PRIMER NAME	PRIMER SEQUENCE	PRODUCT SIZE
CG6422 F1	GCAACGCCGTTCGCGTGAAAAGTGAC	F1/R1 354 bp
CG6422 F2	GCAACAAACTCTGCCTGCCTTGTG	F2/R1 216 bp
CG6422 R1	CCTGCGAAATCCCGACATAAATCGAATC	
CG6422 F3	CAGCACTGGTTTGGCGCAATCGGTAC	F3/R2 241 bp
CG6422 R2	CGCTTCCGAAGCCGTCACTTTTCAC	

CG6422 F4	CAATACCTTTCACAAGACCGACCATC	F4/R3	254 bp
CG6422 R3	CAAGCAGCCGAACGCAACCACTCAC		
CG6422 F5	GGTGCTCAACCGATCACGGCAATAAG	F5/R4	245 bp
CG6422 R4	GTGACGCAGCGTTCCGTTAGGCACATC		

Table 1. Representing the designed primers with names, sequence and product size.

3.2.2 Single Fly PCR

Requirements: PCR Mastermix - Water - 42 µL; 10x Buffer – 5 µL; dNTPs – 1 µL ; Taq Polymerase enzyme – 0.3 µL; Primers – 1 µL each. Dispense 47 µL of the mastermix into each tube.

The single fly PCR involves using a fly as a source of DNA directly.

Procedure:

A single male fly (because female flies might have stored sperm which can act as a DNA aberration) was selected and put in a pcr tube. The fly was killed in the tube by placing it at -20°C for at least 30 min. 200 μ L of isopropanol was added and the tube was incubated for an hour at RT. The tubes were then put in a Speedvac for 30-45 min at 65°C or till dry. The forward and reverse primers were individually pipetted on the walls of the tube without mixing them. The mastermix was added to the tubes, while simultaneously washing down the primers on the walls into the mix. The PCR mix with the fly was placed into the thermocycler after a quick spin.

PCR Program

30” 94 ^{o}C (Initial Denaturation)

30” 94 ^{o}C (Denaturation)

40” 56-60 ^{o}C (Annealing) } 40 Cycles

45” 72 ^{o}C (Extension)

120” 72 ^{o}C (Final Extension)

∞ 4 ^{o}C (Final Hold)

3.2.3 Agarose Gel Electrophoresis

To run the PCR samples Agarose gel was prepared at a 2% concentration.

Requirements: Agarose -2g, Distilled water -100ml, Ethidium Bromide - 5µL per 100 mL, Loading Dye/ Blue Juice (Bromophenol Blue, Xylene Cyanol, Glycerol, EDTA, Tris), 100bp Ladder (Fermentas)

Procedure:

Add agarose to water in a 250mL conical flask. Heat the suspension until the agarose dissolves to get a clear uniform solution. Let the solution cool without solidifying and add the ethidium bromide solution. Place combs in the gel tray and pour the solution after thorough mixing. Let the gel solidify and remove combs. Store at 4°C until use.

The PCR samples were run in TAE buffer (1x) using a loading dye (2 μL) with 10 μL of sample. 4 μL of a 100bp ladder was used. The gel was run at 300V for 20-25min. Then the bands were visualized using GeneSnap (Transilluminator).

3.3 Negative Geotaxis

Knockout mutant flies were tested for neural problems in comparison to wildtype flies using this assay.

Ideal setting for experiments – Room should be at 25°C and light source (should be ideally above).

The assay should be carried out at the same time of the day, as activity of the flies change throughout the day and males and females should be separately tested.

Procedure:

3x 15 appropriately aged flies per genotype (in this case 2 genotypes) were taken and anesthetized with co2 and put in adapted 25ml pipettes to be used as vertical climbing columns. The top and bottom of the pipette were closed with cotton. The flies were then allowed to recover for 30 minutes at 25°C in an incubator before starting the assay.

Pre-Assay training period: the flies were trained once before the assay was started; where the flies were tapped to the bottom (with the same number and intensity of the taps) and allowed to climb for 45 seconds.

Assay: Flies were then again tapped to the bottom and allowed to climb for 45 seconds. Upon whose completion the numbers of flies above the 25mL line and below the 2mL were recorded. This was repeated consecutively for 2 more times for all the 3 sets for each genotype.

Analysis: The climbing ability was then calculated by a performance index (PI) given by –

$$\textbf{Performance Index (PI)} = 0.5 \times (n^{total} + n^{top} - n^{bottom}) / n^{total}$$

N total = total number of flies

N top = total number of flies at the top

N bottom = total number of flies at the bottom

3.4 Larval Brain Staining For DMT-A70 Mutant Flies With 3' Epitope Tagged DMT-A70 Mutants

Requirements: Forceps, PBS, PBT (PBS + 0.1%Triton X), Fix, 1° Antibody [2 types: 1:20 anti- ELAV (in rat) + 1:50 anti- HA (in mouse) + 10% NGS + 0.1% Sodium acetate in PBT]; 2° Antibody: [1:200 anti-rat (Red) + 1:200 anti- mouse (Green) + 10% NGS + 0.1% Sodium acetate in PBT], Glass slides, Cover slips

Procedure:

Selected non-tubby larva and placed them in PBS. Dissected the larva by splitting them in half and inverting the skin over the other forcep by holding the mouth hooks (Basically turning the larva inside out). Fixed the larva by adding 200 μL of the fix for 30 minutes. Washed with PBT for 3x for 10-30 min each. Added the 1^{o} antibody and keep it 4°C overnight. Removed antibody and washed the larva with PBT 3x for 10-30 min. Added the 2° antibody and kept in dark for 4 hours. Washed after removing 2° antibody. Checked staining under fluorescent microscope. If the staining was satisfactory, the brain attached to the head was neatly and carefully removed, clearing the eye and leg discs without damaging the brain.

The brains were neatly aligned on the glass slide. The mouth hooks were placed on four corners on the slides around the brains. Vectashield was added and the coverslip was placed on the mouth hooks, carefully not squashing the brains. Viewed under the fluorescence microscope and then transferred to a confocal microscope.

4. RESULTS

4.1 Creating A Knock Out In CG6422 By Imprecise Excision Of A P- Element

The fly stocks were used to put up crosses as per the crossing scheme to get knock out mutants for my gene of interest that is CG6422. The gene CG6422 has another gene sequence CG3115 being transcribed in the opposite direction. The gene CG3115 might have implications on the knockout, which are yet unclear.

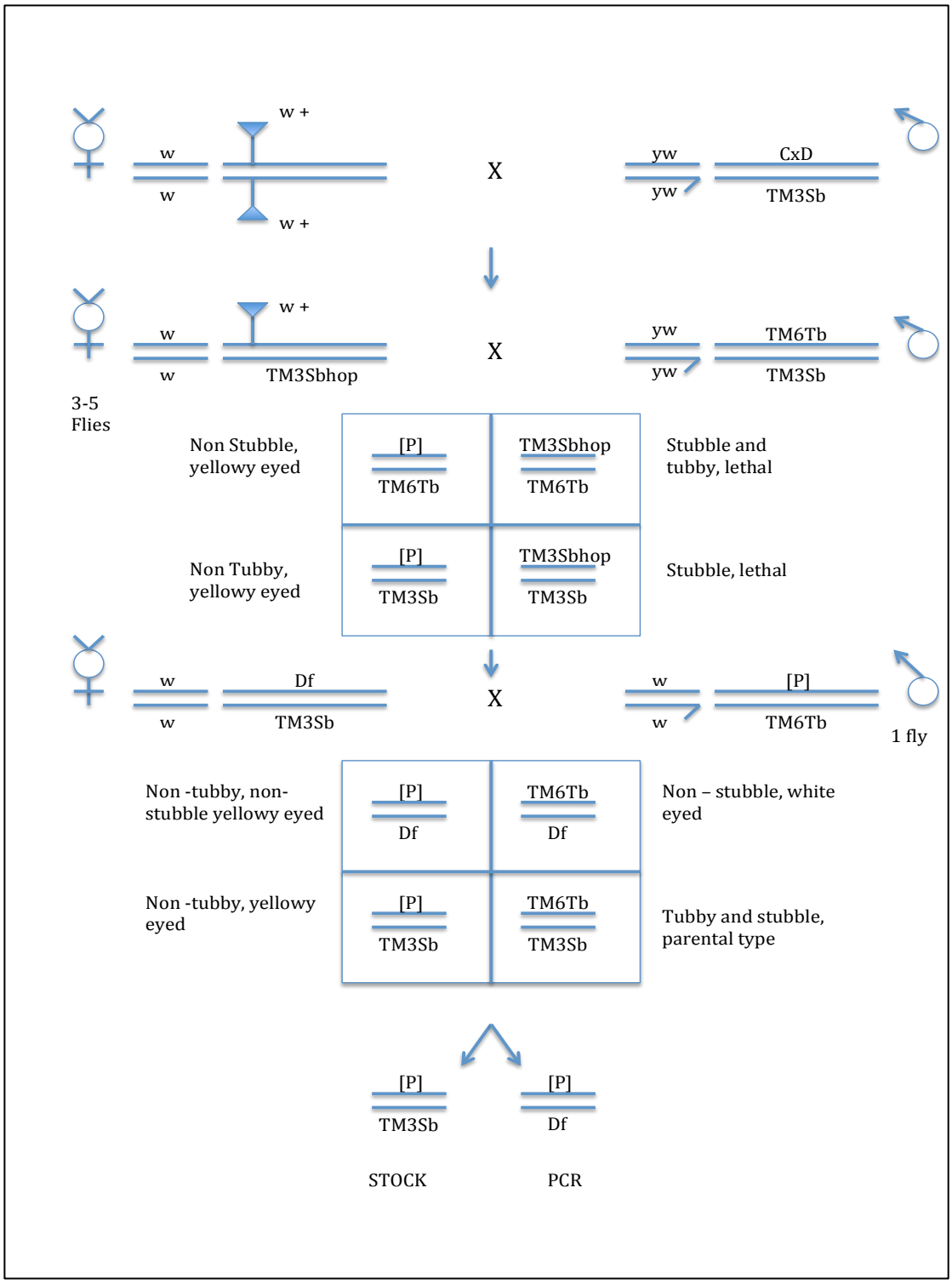


Figure 6. Crossing scheme depicting the entire experiments for getting knockout flies. w – white eyed, yw- yellow eyed, w+ - transposon, Sb- stubble, Tb – Tubby, [P] – P element jumpout, Df – deficiency. The crossing scheme depicts how the genetic crossing over of specific flies was used to achieve a knockout mutant.

The flies from the stocks were used to put up crosses as mentioned above (Figure.6). The flies were distinguished using various phenotypic markers and crossed with female virgins to get the first generation of offsprings. The flies from these crosses can be collected till 17 days after the first fly comes out after which the second generation flies start emerging.

The crosses were labeled by number 1,2,3 etc. and each time they are flipped they are labeled as 1A, 1B etc. So when a fly is collected for the single male crosses it is labeled for e.g. as 1Ai which means a fly from the first vial of the cross. If another fly is collected from the same vial it is labeled as 1Aii.

4.3 Single Fly PCR

4.3.1 Primer Check By Fly PCR

CG6422 is located between genes *smg6* and *bai.* The gene has another gene CG31115 in an opposite transcription frame between 862,666 to 864,971bp of the CG6422 gene. It has been known to have a molecular function similar to S-methyl-5-thioadenosine phosphorylase activity and might be involved in nucleoside metabolic activity (Graveley *et. al.*, 2011; Flybase)

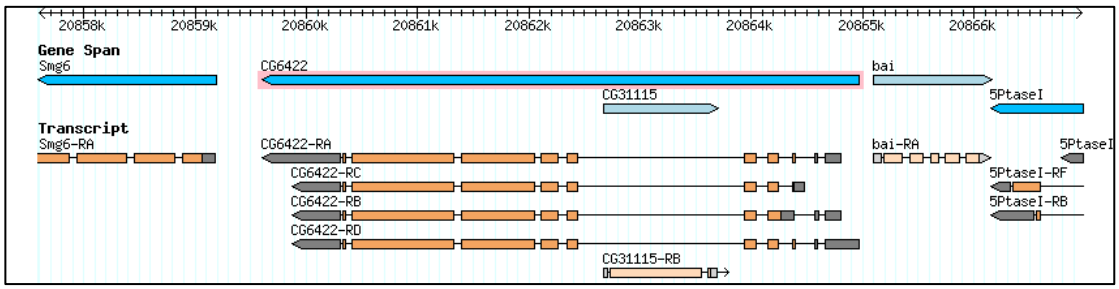


Figure 7: The above figure describes the general position of the gene CG6422 within the fly genome. The highlighted arrows in blue depict the gene, with the direction of the arrow depicting the direction in which the gene in transcribed. The arrows below

in orange depict the RNA transcripts of the gene. The grey parts highlight the gene beginning and end alongwith direction of transcription.

Primer pairs as per figure 5 were used to detect the gene knockout. Below are the various PCR results:

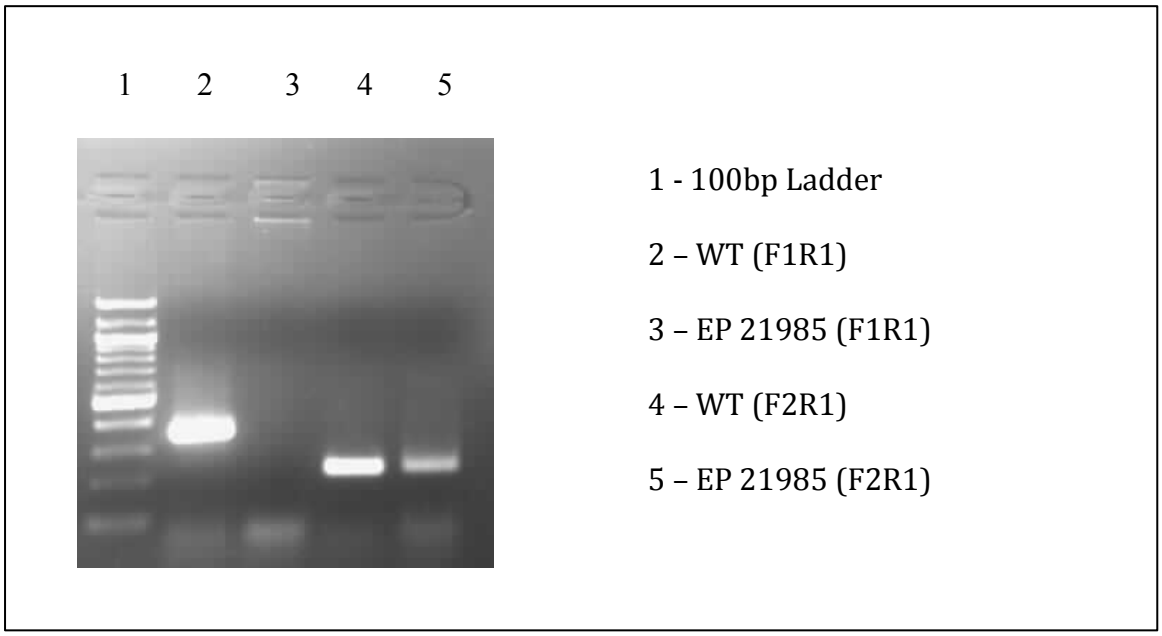


Figure 8: Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product.

Primers were checked using wild type flies (WT) and P-element containing flies (EP 21985) for deciding the correct primers to use for screening. The primers that did not go over the transposon were selected to screen out flies with imprecise jumpouts (transposon excision) (Fig.9)

4.3.2 Screening Fly By PCR For Jump Outs

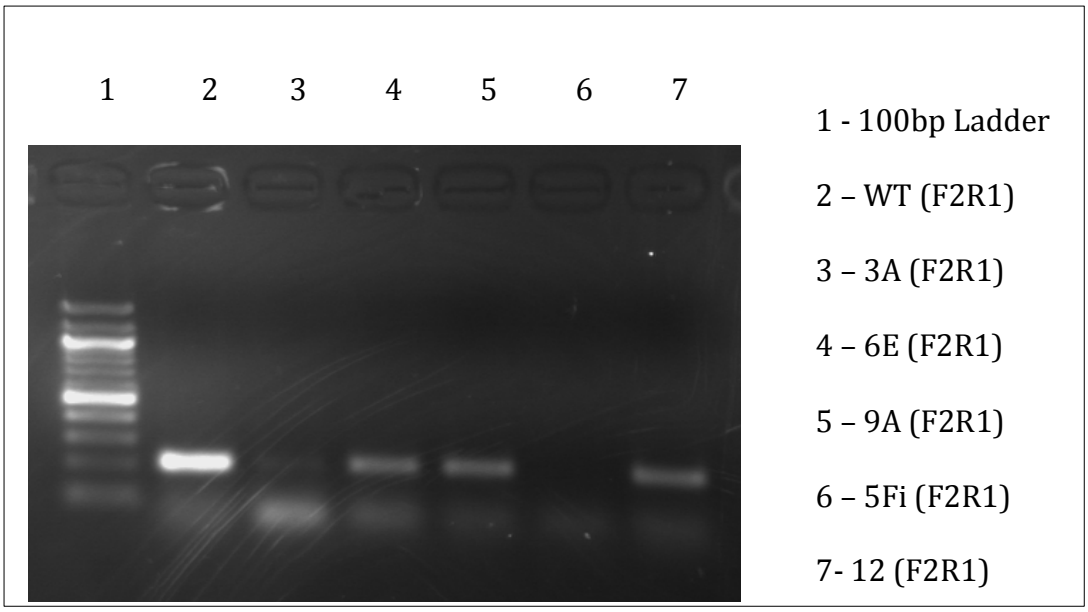
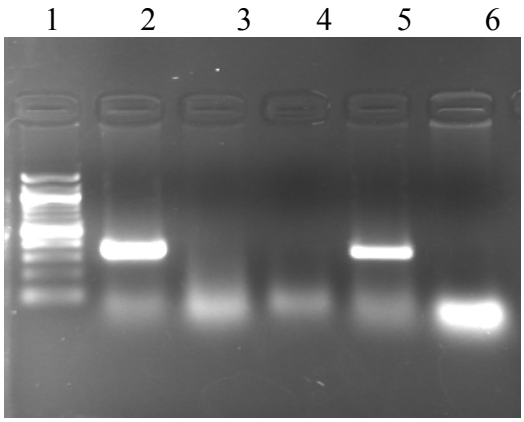


Figure 10. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Absence of an amplified product reports a positive result for this experiment.

Male non-stubble non-tubby flies were selected for screening jump-outs. Some of them gave bands, which were indicative of precise jump outs while others without bands were indicative of imprecise jump-outs. The ones with imprecise jump-outs were selective and repeated with the same as well as other primer pairs for confirmation (Fig 10).

4.3.3 Repeating Testing With Flies

PCR was repeated for probable jump-out fly crosses by retesting the flies with all the other PCR primers designed as per the picture depicted before.



Lanes

1 - 100bp Ladder

2 – WT (F1R1) – 354bp

3 – 3A (F1R1)

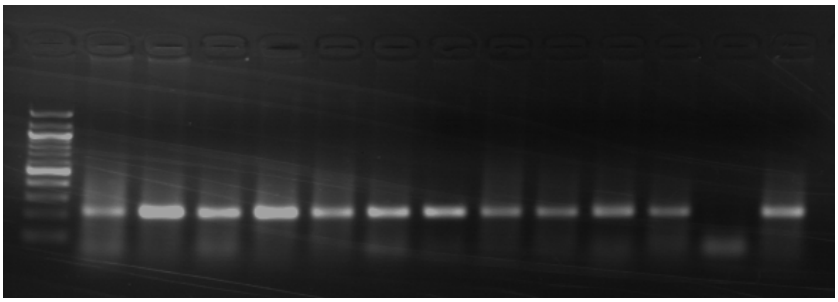
4 – 5Fi (F1R1)

5 – 1Gi (F1R1)

6 – EP 21985 (F1R1)

Figure 11. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Confirmatory absence of a band against a negative control.

The PCR using the primers F1R1 recorded absence of bands for two of the probable fly crosses along with the negative control (i.e. EP 21985) (Fig.10)



Lanes

1 - 100bp Ladder

2 – WT (F2R1)- 216bp

3 – 1Hii (F2R1)

4 – 5Gi (F2R1)

5 – 9Bii (F2R1)

6 – 9Ci (F2R1)

7- 11Ci (F2R1)

8 – 13Bi (F2R1)

9 – 2Hi (F2R1)

10 – 3Hi (F2R1)

11 – 1Gi (F2R1)

12 – 9A (F2R1)

13 – 5Fi (F2R1)

14 – EP 21985 (F2R1)

Figure 12. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Using a different combination of primers to check for imprecise excision.

The PCR with primers F2R1 flank the right hand side of the transposon recording any imprecise jump –outs. As seen above along with the probable flies other fly crosses were screened as well resulting only in one correctly imprecise jump-out (Fig. 12).

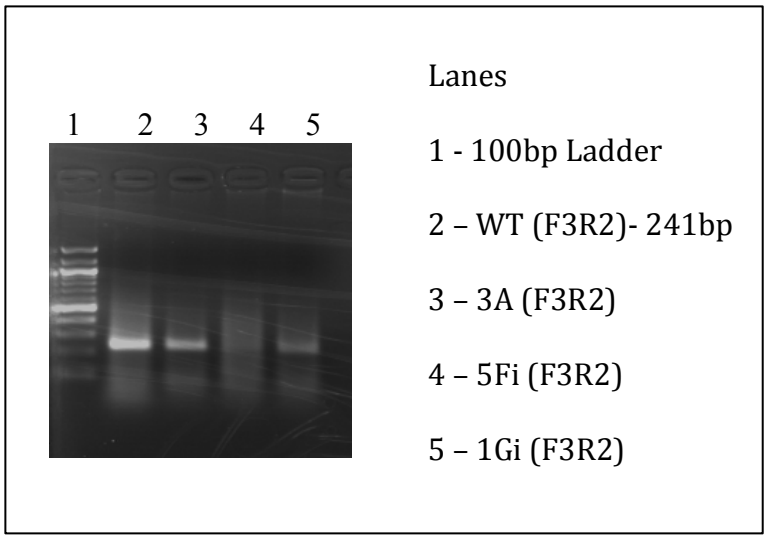


Figure 13. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Absence of a band confirms the presence of an excision from the desired gene.

The primers F3R2 are on the left side of the transposon, absence of a band in the 4th lane sample, confirms the presence of a considerable length of the gene being jumped out (Fig.13).

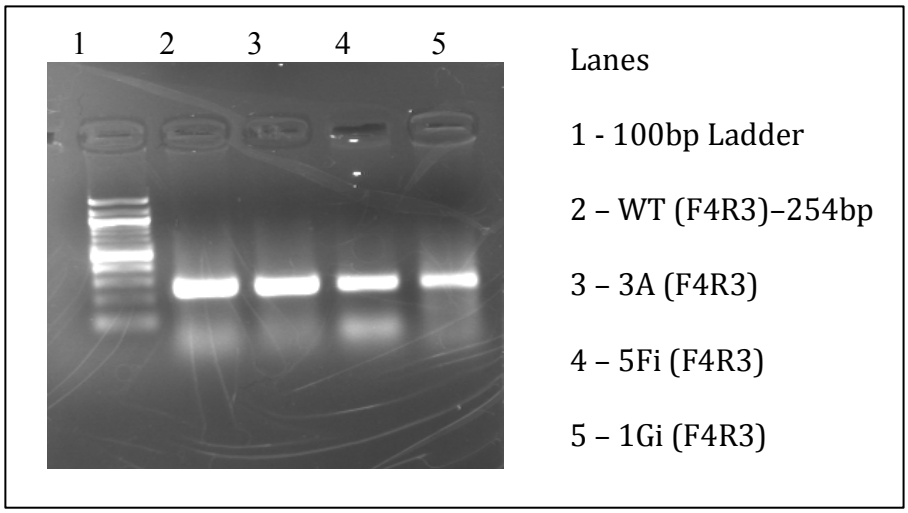


Figure 14. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. PCR to check the probable length of the gene fragment, which was excised.

F4R3 primers were used to detect the length and probable region of the jump-out, presence of bands in all of them shows that the region of the gene has not jumped out (Fig.14).

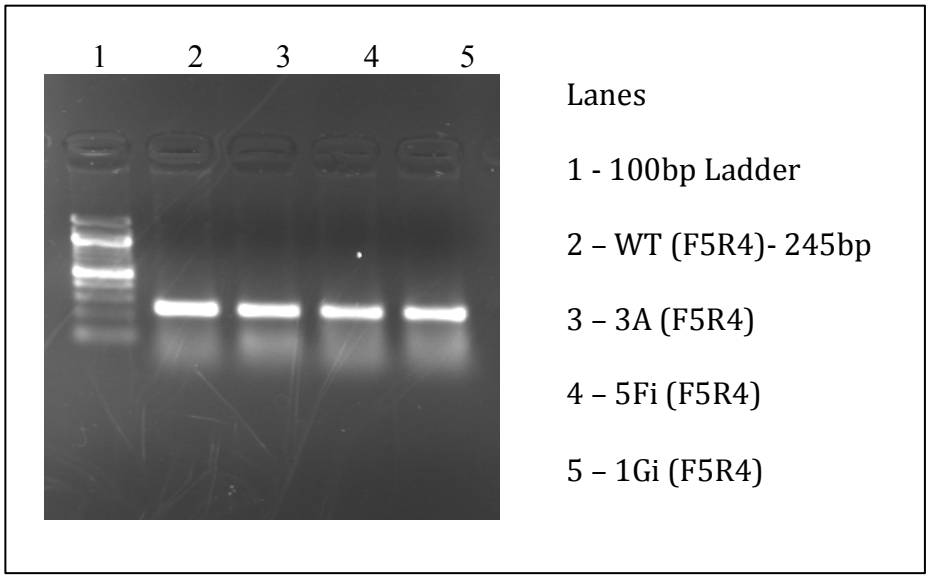


Figure 15. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product using different primer combinations to map the excision.

All the above-mentioned PCR results were conclusive of an imprecise jump-out for one cross (Fig.15). The flies from these vials were selected to put up a stock and to map out the jump out length. Stubble flies were selected and crossed with virgin deficiency flies in absence of occurrence of virgin stubble flies along with a stock for the jump out upon deficiency flies (P/Df) for further testing.

4.4 Frequency Of Jump Outs

The frequency of flies jump out was calculated as per the number of flies coming out per vial and to that the number of jump out flies coming out.

Before that the frequency for the single flies used to setup the single male fly crosses were calculated to be 1.42%, which is a good frequency level.

The frequency for precise jump out flies was 0.77% as opposed to the imprecise fly frequency which came up to 0.52%, which is a very low percentage frequency attributed to the difficulty in phenotypic distinguishing and also the possible low activity of the P-element transposon on the fly.

4.5 Bioinformatics Results

The CG6422 gene in Drosophila is orthologous to the YTHDF1 in Homo sapiens. The gene name stands for YTH domain family protein 1 which is 559 amino acids and contains one YTH domain. It forms two isoforms by alternate splicing as opposed to the 4 isoforms of CG6422 (A to D). Although the genes are orthologous to each other they show very less similarity in sequences in the BLAST results. That is it shows 68% identity with 2% query coverage and negligible E value. The YTHDF1 gene is present on the 20^{th} chromosome in humans. The YTH domain containing proteins haven't been structurally characterized in a crystal but have been characterized in a solution as shown in figure 16.

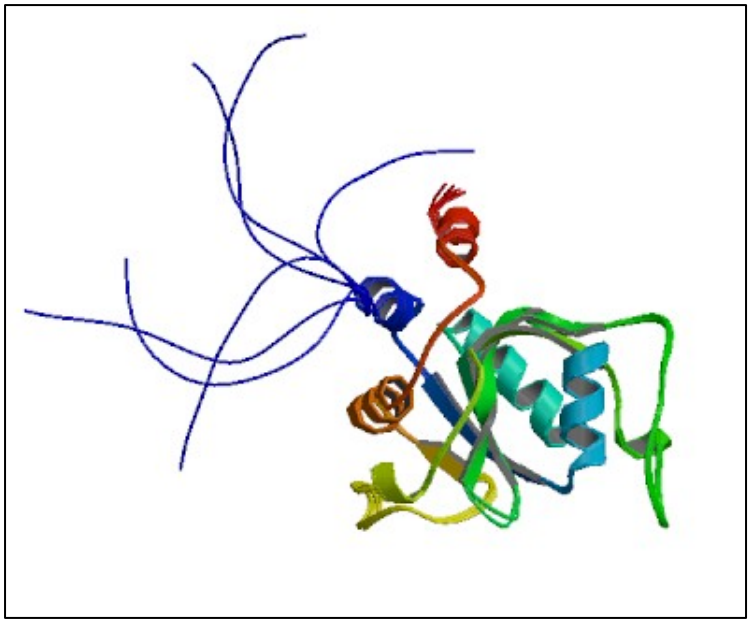


Figure 16. Protein structure of YTH domain containing protein in solution. (PDB)

The amino acid sequence of CG6422 was compared with another drosophila YTH domain containing protein that is YT521-B. The BLAST result of the two sequences does not show much identity (around 29%) that might mean that the proteins although having YTH domains might not be exactly with the same structure or function.

4.6 Negative Geotaxis

The graph below represents the negative geotaxis variation between the wild type and the jump-out deficiency flies. As seen below the flies with jump-outs have lower performance indexes in comparison to the wild type flies. Although the major difference is seen in case of female flies compared to male flies, the knockout female flies are more affected by the jump-out (Fig.17).

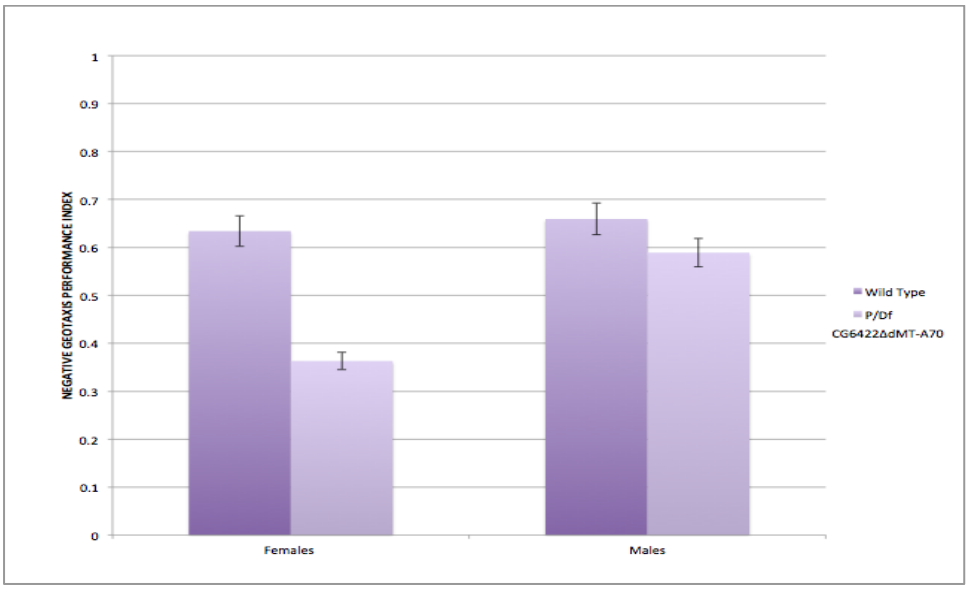


Figure 16. Negative geotaxis performance index; Females: Wild Type (WT) and P/Df and Males: Wild Type (WT) and P/Df (CG6422ΔdMT-A70).

4.7 Epitope Tagged Proteins From 3' DMT-A70 Mutants

Larval brains staining from epitope tagged non-tubby larva of dMT-A70 are shown below. The stains involved are for ELAV (red) and HA (green); both are localized in the nuclei (Fig18).

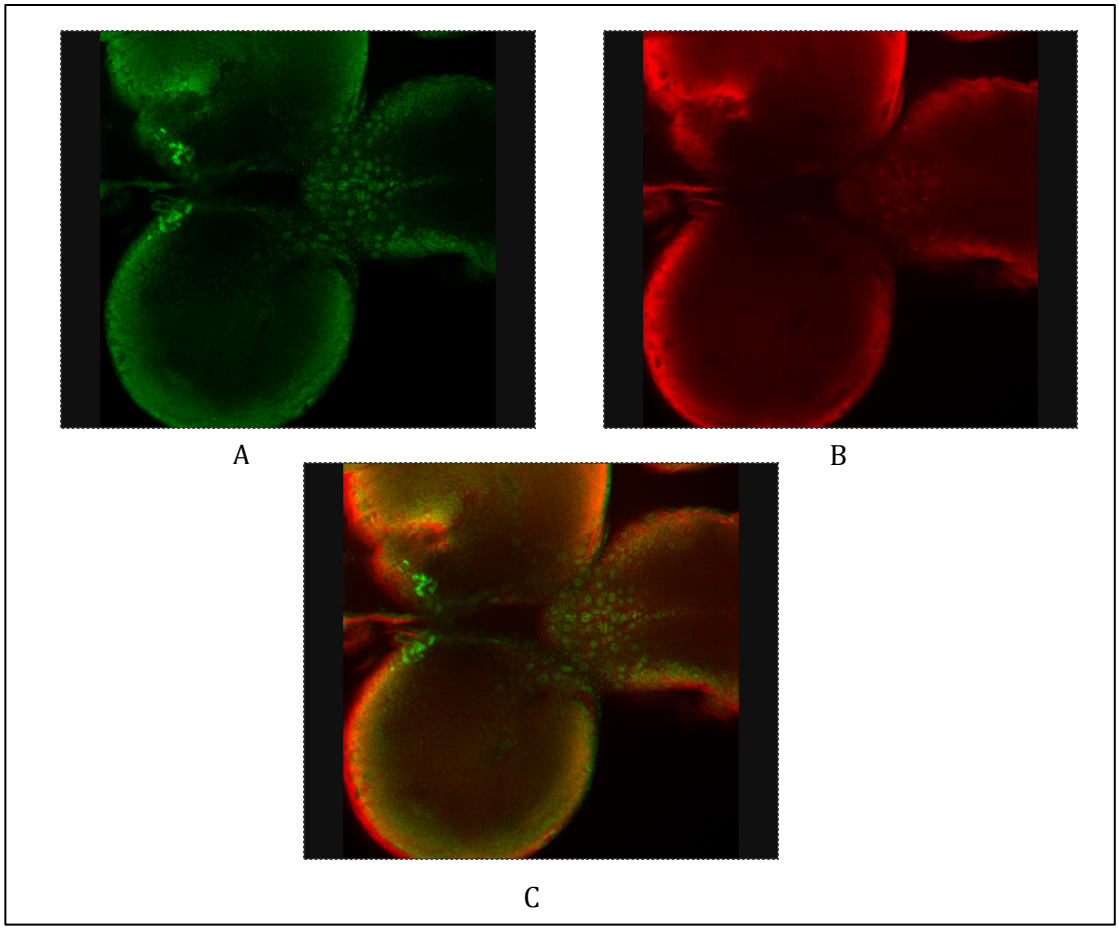


Figure 17. A. Larval Brain with HA staining. B. Larval brain with ELAV staining. C. Superimposed images containing both HA and ELAV stains.

The staining does show nuclear localization strongly in several cells but due to the absence of clear nuclear staining for ELAV it cannot be deciphered as to whether the developing cells are neurons or not. The results for the above mentioned cannot be considered conclusive due to improper diffused staining of ELAV.

5. DISCUSSION

The aim of this was to study internally methylated RNA sites and to understand the plausible effects of absence of YTH domain containing proteins that interact with these methylated sites for regulation and the development of neurons in drosophila. As per the experiments conducted one single jump-out mutant was found among 118 crosses setup for the producing knockout flies..

The reason for such a low number of jump-out flies could be the presence of the position effect in the crosses with the transposon and T36 flies. The flies used to setup single male crosses should have been white eyed as per the crossing scheme but opposite to expectation the flies that came out have eye colors which were very light yellowy almost to white with variable pigmentation. This lead to the conclusion that the possible occurrence of position effect and the presence of additional transposons might have caused the occurrence of this eye color. Therefore, after this when flies were used for screening, the deficiency and tubby have very minute differences in phenotype which might have been a key factor to the fact that many samples gave a positive result (that is the absence of a band after PCR for imprecise jump out) but gave a negative one when retested. This could have been one of the major drawbacks for finding only one jump-out. It could also have been that the transposase activity is very low which was the reason behind the jump-outs being low in number.

The jump-out flies, which were found to be positive were used to put up stocks and expressed a level of neural disruption as experimented via the negative geotaxis. The female flies in the jump-outs show a lower performance index as compared to males.

This might be because the P-element flies have a transposon, which is propagated through the female line, which could be a reason for the strong neuronal dysfunction in females compared to males. The bioinformatics studies of the proteins with YTH domain involve CG6422 and YT521 B mainly which although are not identical in anyway have similar domains and are supposedly having similar regulatory functions in methylated RNA for neuronal development.

Further studies on the same project would involve, mapping the imprecise jump-out and therefore getting to know the extent of the gene disruption. This could be taken further by developing a complement for the knocked out gene to deduce the effects without the gene and on addition of the complement to the cells on neuronal development in flies from all stages (larval to adult).

Studies for sexual development and flight hampering could also be performed. A negative geotactic comparison with the dMT-A70 mutants wasn't performed due to shortage of flies, which died over a period of 15-day incubation outside for slow growth.

The dMTA-70 epitope tagged transgenes were basically done to study the development of larval brain and whether or not the developing calls at the lobes were neurons or not. This would have lead to the identification of the fact that absence or presence of the methylase enzyme affects regulation and development of neurons. But due to the diffused staining of the ELAV protein antibodies no conclusive results could be drawn from it. At best repeating the experiment could carry studies forward.

The next step in this study after mapping the gene could be producing a complementary transgene of the jump-out and then microinjecting these into the mutant embryos or larva to see effects on development and adult neuronal status. This could give conclusive evidence about the functional importance of YTH domain containing proteins along with their role and level of interaction with methylated RNA.

The gene could also be used to produce proteins via recombinant techniques and added to the larva to view direct effect of the protein on the larval development, as it should interact with methylated mRNA therefore regulating neuronal growth. This project has made me think a lot on the newer avenues of neuroscience and neural development and I have gained a lot of new experience through it.

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LIST OF FIGURES AND TABLES:

mRNA processing types are given: artificial gene on top and mRNA isoforms below. Alternative mRNA processing is depicted by dotted lines while constitutive ones with solid lines. Adenosine to inosine editing (A to I), have editing site complementary sequences (ECS) present in the introns with edited site in the exon. (Soller, 2006)

Figure 2. The structure of various types of methylation in RNA is shown. The green ball indicates methylation in the cap and the pink ones indicate internal methylation. (Jia *et. al.*, 2013)

Figure 3. m6A – seq capture of modified RNA fragments exposes an enriched motif. a. Diagram representing the method used to detect m6A methylation using anti-m6A Ab and immunoprecipitation.b. Consensus motif representing by the sequence logo. c.Density plot describing the distribution of distance between the peaks of m6A/control fragments and the nearest m6A consensus motif (RRACU) (Dominissini *et. al.*, 2012)

Figure 4. A. Sex differentiation with male flies having sex combs, penis and a genital arch while females have a vaginal plate and virgins have a meconium spot on the abdomen. B. Multitude of phenotypes from body colour, bristle type, wing type and shape, larval size. C. Difference between a wild type bristle and stubble bristles. (Song *et. al.*, 2007)

Figure 5. The primer pair positions are depicted in the diagram with sizes of amplified products.

Figure 6. Crossing scheme depicting the entire experiments for getting knockout flies.

Figure 7. The above figure describes the general position of the gene CG6422 within the fly genome. The highlighted arrows in blue depict the gene, with the direction of the arrow depicting the direction in which the gene in transcribed. The arrows below in orange depict the RNA transcripts of the gene.

Figure 8. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product.

Figure 9. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Absence of an amplified product reports a positive result for this experiment.

Figure 10. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Confirmatory absence of a band against a negative control.

Figure 11. Agarose gel depicting bands of amplified DNA with respect to specific primers used with reference to a 100bp DNA ladder to compare the size of the amplified product. Using a different combination of primers to check for imprecise excision.

Development of a phenotypic high throughput-screening assay to identify novel molecules that inhibit Mycobacteria.

By

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Project 2 - MRes in Molecular and Cellular Biology

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List of Abbreviations

% - Percentage

°C - Degree Celsius

ACP - Acyl carrier protein

ADH - Anti Diuretic Hormone

AG - Arabinogalactan

AIDS - AutoImmunoDeficiency Syndrome

BCG - Bacillus Calmette–Guérin

DA - Dopamine

DAT - Dopamine Active Transporter

DMSO - Dimethyl sulfoxide

DOTS - Directly Observed Therapy – Short Course

EMB - Ethambutol

ER - Endoplasmic Reticulum

FAD - Flavin adenine dinucleotide

FDA - Food and Drug Administration

FLD - First Line Drugs

G + C - Guanine + Cytosine

GFP - Green Flourescent Protein

g - Grams

HIV - Human Immunodeficiency Virus

hrs - hours

HTS - High Throughput Screening

KAS - β-ketoacyl synthase

LAM - Lipoarabinomannan

LM - Lipomannan

M.I.C - Minimum Inhibitory Concentration

MA - Mycolic Acid

MAC - Mycobacterium Avium Complex

MDR-TB - Multi Drug Resistant Tuberculosis

mg/mL - milligram/ millilitre

mM - millimolar

NAD - Nicotinamide adenine dinucleotide

NADH - Nicotinamide adenine dinucleotide (reduced)

$O.D_{600}$ - Optical Density at 600 nanometers

PDIM - Pthiocerol Dimycocerosate

PIM - Phosphotidyl Inositol Manoosides

PPD - Purified Protein Derivative

PTP - Phosphotyrosine phosphatase

RLU - Relative Light Unit

RNA - Ribonucleic Acid

rpm - Revolutions per minute

SL - Sulfolipids

SLD - Second Line Drugs

SSC - Short Course Chemotherapy

TB - Tuberculosis

TDM - Trehalose Dimycolate

TLR - Toll Like Receptors

TMM - Trehalose Monomycolate

TNF - Tumor Necrosis Factors

UDP - Uridine Diphosphate

VGCC - Voltage Gated Calcium Channels

WHO - World Health Organisation

XDR-TB - Extremely Drug Resistant Tuberculosis

μg/mL - Microgram/ Millilitre

Abstract

Tuberculosis is a major cause of death around the world. It has afflicted mankind for centuries with its inherent property of remaining dormant within the host system. With development of new drug regimens by health administrators all over the world the disease was thought to have been under control until the emergence of newer strains of Mycobacterium, which are resistant to antibiotics prescribed to combat it. Due to the contagious nature of the disease the drug resistant strains have started becoming more prevalent leading to problem of treatment due to dwindling treatment options. This calls for new drug discovery in the area, which could lead to potential for newer drug regimens for treatment of drug resistant strains. In order for quicker drug or antibiotic discovery, synthesized compound libraries have been screened through highthroughput screening, reducing the gap between the compound discovery and activity helping to process drug libraries quickly to get the possible novel drugs in use. The aim of this project was to develop a new and efficient assay to be used for highthroughput screening of compound and FDA (Food and Drug Administration) approved drug libraries. The assay developed was a phenotypic assay based on fluorescence level detection correlating to bacterial survival in M. smegmatis (mc^2155) strain. The results obtained from the work done have led to some novel and promising drugs successfully screened using the newly designed and optimized assay. Further work has to be done on the mode of action and dose response activities of the "hits" achieved from the screens.

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1. INTRODUCTION

1.1 History

Koch discovered the causative agent of Tuberculosis more than 100 years, which is *Mycobacterium tuberculosis*. The oldest remains found of tuberculosis were from bones of skeletons aged 5400 years, which means the disease was prevalent during that time. The bones excavated suggested that the person was suffering from Pott's disease, which is an extra pulmonary form of tuberculosis deforming the spine (Curbezy *et. al.*, 1998). Tuberculosis was also common in Greece, as Hippocrates well recognized the disease "phthisis" and could identify its clinical symptoms. He described it as consumption in his "Book I, Of The Epidemics" and wrote about how it mostly affected young adults proving fatal to more people than any other disease. (T.Daniel, 2006). Later during the 18^{th} and 19^{th} century consumption was considered incurable and possibly a hereditary disease as it prevailed mostly among families through generations while others debated it to be occurring due to stress and poor nutrition (T.Daniel, 2006). Speculations were still ongoing even after Dr. Jean-Antoine Villemin, proved the contagious nature of the disease by successfully transmitting the infection from humans to rabbits (J. F Murray, 2006). It wasn't until Robert Koch presented his famous lecture in Berlin, 1882 on *Die Aetiologie der Tuberculose* that the exact cause of the disease was recognized. A few years later, he isolated a substance from the bacilli which when injected gave an immune response therefore proving itself as a probable diagnostic, the substance was called tuberculin (ref). The test was further improvised over the years, such as Charles Mantoux

introducing the protein intracutaneously using cannulated needles and Florence Seibert developing purified protein derivative (PPD) (T. Daniel, 2006). It still remains one of the widest known afflictions to mankind due to its resilient nature and clever strategies of survival, which circumvents the host immune system. This makes *Mycobacterium tuberculosis*, the causal organism of tuberculosis a very successful pathogen (I. Smith, 2003; Mukhopadhyay *et. al.*, 2011).

1.2 Classification

The origin of *Mycobacterium tuberculosis* is thought to have been from a progenitor species of actinomycetes existing in soil, which evolved to live reside in a mammalian host. The domestication of cattle around 25,000 years ago would have lead to the transmission to humans by evolutionary development into *M. tuberculosis*. Early hypothesis of the development of *M. tuberculosis* from *M. bovis* was falsified in view of recent genomic experiments, which prove that *M. tuberculosis* developed from other *Mycobacterial* complexes such as *M. africanum*, *M. microti*, and *M. canetti*, as well as *M. tuberculosis* and *M. bovis* which affect both humans and animals equally *(*I. Smith, 2003*)*. Due to the high level of similarity within the housekeeping genes between the *M. tuberculosis* complex it is thought that the species underwent an evolutionary slow down around 15000-20,000 years ago (Brosch *et. al.*, 2002).

Mycobacterium complex is classified under the family *Mycobacteriaceae* that is reflected by the presence of high percentage long chain fatty acids called mycolic acids in its cell wall. It is further encompassed under the order of *Cornybacterineae* defined as an acid-fast bacilli with a high G + C content. The order is *Actinomycetales*

and phylum *Actinobacteria*, which are defined by being aerobic gram positive bacteria with a genetic composition rich in guanine and cytosine and cell walls containing peptidoglycan and mycolic acid (Minnikin, 2002).

1.3 Pathogenesis/Immunology

The infection by *Mycobacterium tuberculosis* is transmitted from person to person by droplet nuclei about 1-5μm in diameter, which are dispersed in air by coughing, sneezing, speaking and singing. The bacilli in these nuclei are airborne for a long period of time but this might not necessarily mean that people coming in contact will be infected by it. There are four major factors governing the infection, a. number of organisms expelled by an infected person in the surrounding, b. concentration of the bacilli within that volume of air and the ventilation level in the area, c. length of exposure to the contaminated air by a person and d. the immune status of the person. The droplet nuclei when inhaled by a person transport the bacilli via the pharynx to the bronchial tree and to the bronchules or alveolus. The establishment of the infection depends on the immune strength of the person. *Mycobacterium* is not known to have any endo – or exo-toxins therefore do not cause an immediate immune response, especially if the bacterial load is very low. The bacteria resides within the alveoli and multiplies over a period of few weeks to a bacterial count of 10^3 - 10^4 which then triggers the immune response in the host. The primary immune response in a host is lead by attack of the alveolar macrophages on the bacilli. The tuberculosis bacteria has a special mechanism of survival unlike other bacterial pathogens, therefore instead of evading the macrophages the bacilli seeks entry into the macrophage phagosome utilizing several surface receptors and once inside it modifies

the maturation of the phagosome, increasing its survival percentage (Dunlap *et. al.*, 1999; M.S Glickman and W. R Jacobs, 2002).

Invasion of macrophages and phagocytosis of the bacilli releases an inflammatory reaction causing recruitment of mononuclear cells to form granulomas. Granulomas are mainly epicenters of infection containing the infected macrophage at its nuclei surrounded by foamy giant cells, lymphocytes on the periphery to contain the infection. While within the macrophage the bacilli applies various strategies such as not allowing the fusion of the lysosome with the phagosome or acidification of phagosome, resistance to killing by oxygenated metabolites. The bacteria, therefore counters the microbicidal activity of a macrophage to form an environment to suit its own survival (J.Basu, 2004). The struggle between the bacterial survival and the immune system continues forming what is called a caseating tubercle, where some of the bacilli come out into the extracellular mileu of the cellular debri. The caseating necrosis is still surrounded by T cells and activated macrophages to contain the area of infection. In case of immunocompetent hosts with a robust immune system the activated macrophages and cytotoxic T cells are efficiently engaged. While for hosts with a weaker immune system the activated macrophages are not efficiently supplied which leads to non-specific destruction of lung tissue. The infection can necrotize into the blood vessels, which disseminates the bacteria to other parts of the body leading to extrapulmonary tuberculosis. If the infection necrotizes in the bronchiole the host can then actively transmit infection to other people via droplet infection (Curtiss And Haydel, 2003).

Statistically most healthy people have an asymptomatic infection of mycobacterium tuberculosis, which becomes symptomatic only in 10% of cases and remains latent for

90% of the time. Clinically latent forms of tuberculosis can be defined as infected people who are not symptomatic and cannot spread the disease. They can only be detected by a positive tuberculin test. A person with a delayed type hypersentivity to the PPD test without actual symptoms of tuberculosis is said to have latent tuberculosis.5-10% of people with latent infections develop an active infection during immunosuppressed conditions caused due to chemotherapeutic stress, other disease conditions and age (Curtiss and Haydel, 2003, Tufariello *et. al.*, 2003).

1.4 Epidemiology

The disease is known to cause death of around 1.5 million people a year worldwide, out of which most of them occur in developing countries. With the increase in the number of immunocompromised people due to the higher cases of HIV and AIDS, around 19-43% of the world's population is infected with *Mycobacterium tuberculosis* (Fleischman and Greenberg, 1998; Flynn and Chann, 2002; Dumlap *et. al.*, 1999) Fig.19. Around 15 million people in the U.S have been diagnosed with tuberculosis in the past few years (Lawn and Zumla, 2011).

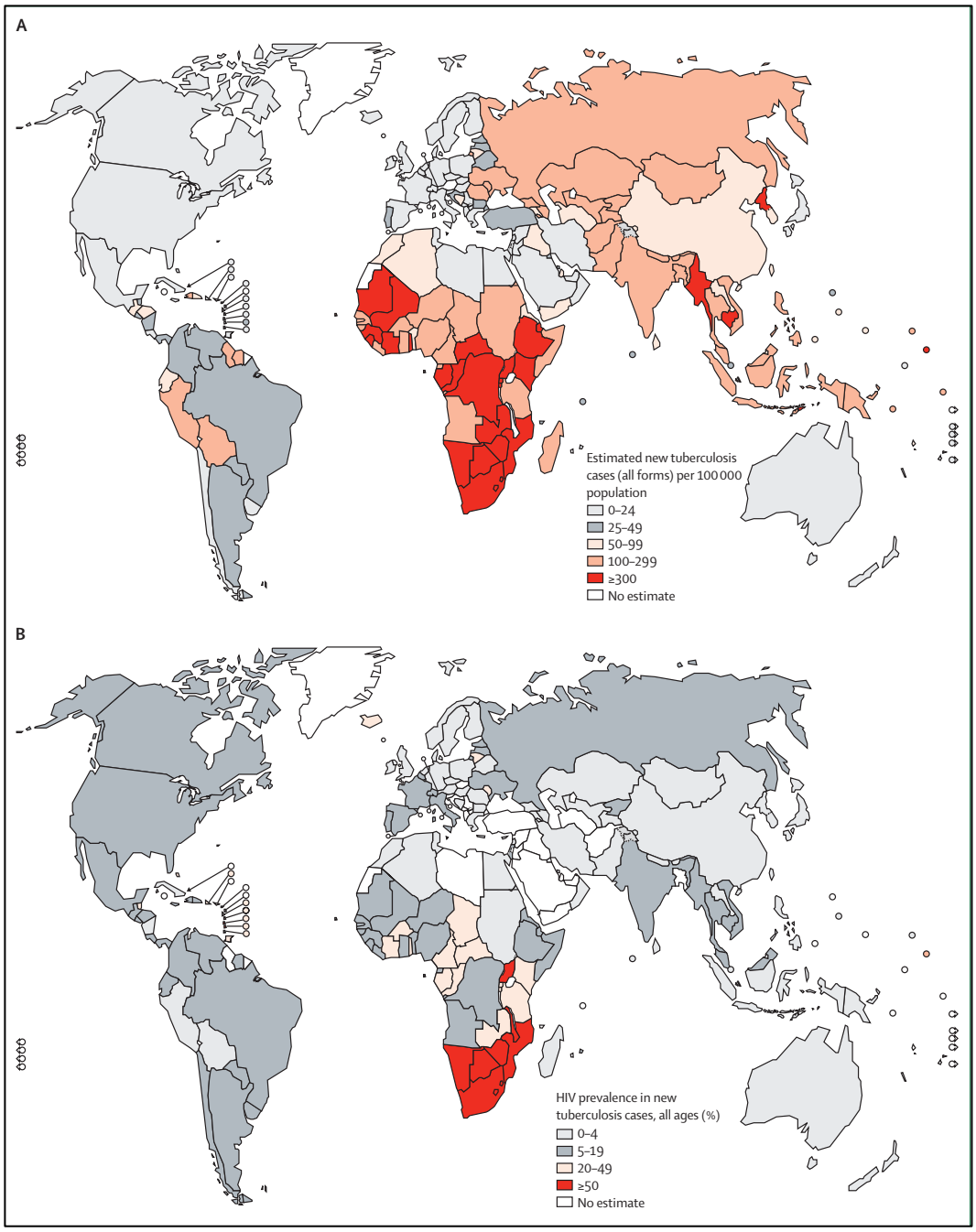


Figure 19: The prevalence and incidence rates of tuberculosis in 2009. A. Estimated rates of tuberculosis; B. Estimated HIV prevalence in new tuberculosis cases (Lawn and Zumla, 2011).

The World Health Organisation (WHO) came up with a strategy to control tuberculosis by a controlled drug therapy program called DOTS (Direct Observed Treatment). The program had five key elements involving political commitment to

areas majorly affected with Tb, detection of case by sputum analysis, short course chemotherapy (SSC), case management by direct observed treatment and recording and reporting of cases. The DOTS program dramatically increased the control of tuberculosis (Gupta *et. al.*, 2001). SSC was the most effective treatment regimen that was planned, wherein patients were treated over a period of 6-8 months with drugs while continuously observing symptoms and microscopic analysis of the sputum. Along with that preventive therapy was suggested to high-risk patients who could easily contract tuberculosis due to a low immune strength such as people with HIV. It was estimated that by 2010 the success of the DOTS program would be observed by 23% decrease in new cases and 26% decrease in the deaths caused by Tb (Dye *et. al.*, 1998).

Unfortunately the development of drug resistance to the first line drugs (FLDs) such as isoniazid, rifampin, streptomycin caused concern due to the development of a new drug resistant strain, MDR-TB (Multi Drug Resistant Tuberculosis) (Blanchard, 1996). Among many things such as lack of knowledge about antitubercular drugs, improper prescription of drugs, non-standardized regimen and poor patient adherence the control of tuberculosis being the major reasons for this strain to develop, which leas to increase in cases of MDR TB with poor therapeutic outcomes. The WHO to deal with treatment of MDR TB established the Green Light Committee for proper use of second line drugs (SLDs) and prevents further drug resistance (Shah *et. al.*, 2007). The SLDs were administered as per requirement after correctly differentiating patients with primary resistance (those patients who had not been treated before but were resistant to FLDs) and patients with acquired resistance (who acquired resistance due to improper FLD regimen). It was clearly observed that patients with acquired resistance were higher than ones with primary resistance (Cohn et. al., 1997).

Unfortunately, cases with resistance to SLDs also started developing, leading to a new strain of XDR TB (Extremely drug resistant tuberculosis). This strain is defined as bacilli resistant to a minimum 3 SLDs as MDR is managed with 4 or more SLDs. Inadequate monitoring of MDR TB, using too few drugs for treatment, poor quality of SLDs, failing adherence to drug regimen could have been the causes for resistance (Shah *et. al.*, 2007; Munro *et. al.*, 2007). The mechanism of acquire drug resistance is not very clearly understood. But there are several studies, which have shown resistance related to chromosomal alterations (S T Cole, 1994) mainly involving mutations in specific genes related to resistance of drugs (Fenner *et. al.*, 2012; Motiwala *et. al.*, 2010). It has also been said that the bacillus gains these mutations sequentially, one drug at a time and may also be able to resist destruction by drugs due to induction of drug efflux pumps (Srivastava *et. al.*, 2010). Drug resistance has also been accredited to horizontal transfer of mobile elements such as plasmids, transposons, integrons that impart resistance to the bacillus (Da Silva and Palomino, 2011).

1.5 Cell wall

The mycobacterial cell wall is made up of two segments, the upper and lower segments. The lower segment of the cell wall is made up of peptidoglycan covering the cell membrane, which is then followed by arabinogalactan, which is bound by mycolic acids. These mycolic acids have long chain meromycolates and short α-chains. The cell wall core is therefore called as the mycolyl arabinogalactan peptidoglycan complex (mAGP complex) as shown in Fig.20. The upper segment of the cell wall is scattered with free lipids intermingled with cell wall proteins,

phosphotidyl inositol manosides (PIMs), pthiocerol-containing lipids, lipomannan (LM) and lipoarabinomannan (LAM) and are known to assist in host immune evasion (Brennan, 2003). Lipids such as trehalose dimycolate/monomycolate (TDM/TMM), pthiocerol dimycocerosate (PDIM) and sulfolipids (SL) are main virulence lipids. There are many cell wall associated proteins such as *lpqH* (19kDa), *pstS1* (38kDa), *tlr2* agonists etc. which play a role in regulating the action of macrophages and dendritic cells along with secreted proteins which act as antigens and can act as drug targets and biomarkers for diagnostics (Wolfe *et. al.*, 2010).

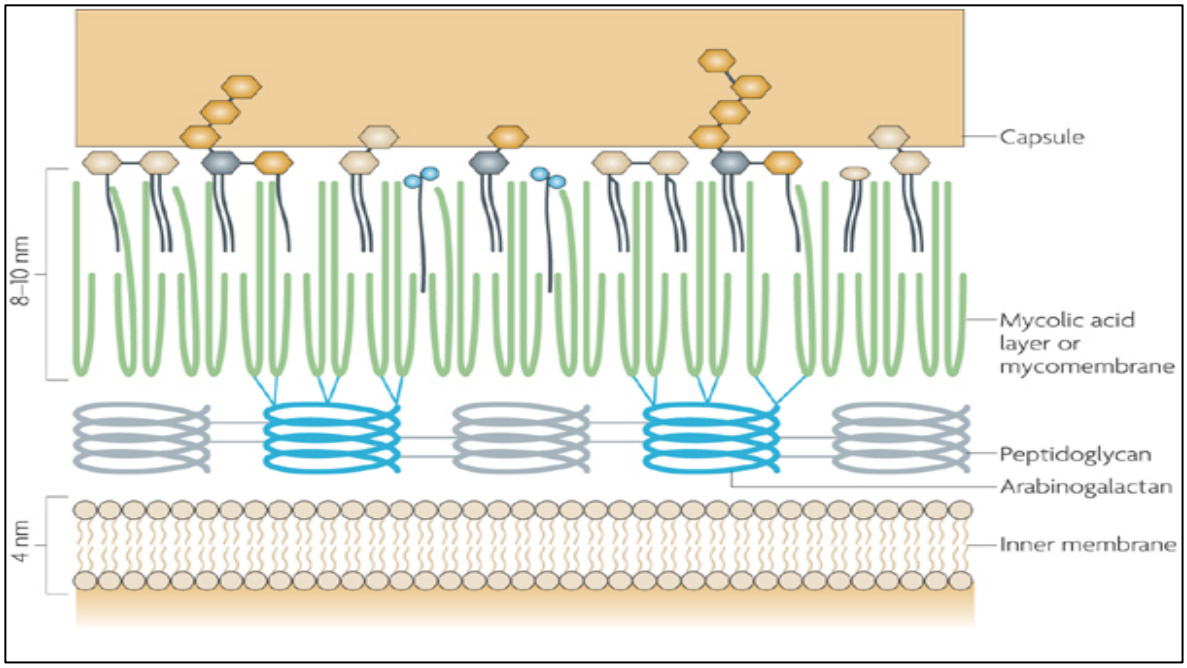


Figure 20: The cell wall components of *Mycobacterium tuberculosis*. (Abdallah *et. al.*, 2007).

Mycobacterium mycolic acids (MAs) play an important role in growth and survival of the bacillus both *in vivo* and *in vitro*. Its different structural properties regulate the manipulation of the host immune system and virulence of the bacillus. The MAs bound to the peptidoglycan cause the inflammatory activation of dendritic cell and

macrophages by interaction via TLR2 and TLR4 receptors. The free mycolates are involved in maintaining the structure of the granuloma, which helps in maintaining the optimal survival condition for the bacillus (Verschoor *et. al.*, 2012). It has also been seen that the mycolic acids stimulate production of antibodies in humans, which can potentially be used for diagnostic and therapeutic purposes. The permeability barrier of the cells produced by the thick waxy cell wall is the major cause of drug resistance in mycobacterium along with other factors such as efflux pumps, which help it to gain resistance against antibiotics. (Niederweis *et. al.*, 2009; Kolattukudy *et. al.*, 1997).

Bacteria from the suborder *Corynebacterineae* which include the *M. tuberculosis* complex (pathogenic) and the *M. smegmatis* (non pathogenic) along with *C. glutamicum* have various liposaccaharides such as LAM and related glycoconjugates varying in each species minutely (Tam and Lowary, 2009) The biogenesis of the cell wall is a complex process involving a series of interconnecting cycles and enzymes, (Acharya and Goldman, 1970) but it is also seen that genes involved in this are essential to the bacteria (Mishra *et. al.*, 2011). Therefore, the understanding of the enzymatic machinery and cell wall synthesis process could lead to novel drug targets and inhibitors to enzymes involved can act as drugs (Mishra *et. al.*, 2011).

1.6 Antibiotics for tuberculosis treatment and resistance

The following table describes the list of antibiotics already in use with specific inhibitory concentrations used for inhibiting the growth of *Mycobacterium*. It mentions the genes whose products are affected by the activity of the antibiotic. Further detailed discussion about the same would be done in this section.

Drug	MIC (mg/L)	Gene	Role of gene product
Isoniazid	0.02–0.2 (7H9/7H10)	*katG*	catalase/peroxidase
		inhA	enoyl reductase
		ahpC	alkyl hydroperoxide reductase
Rifampicin	0.05–0.1 (7H9/7H10)	*rpoB*	β-subunit of RNA polymerase
Pyrazinimide	16–50 (LJ)	*pncA*	PZase
Streptomycin	2–8 (7H9/7H10)	*rpsL*	S12 ribosomal protein
		rrs	16S rRNA
		gidB	7-methylguanosine methyltransferase
Ethambutol	1–5 (7H9/7H10)	*embB*	arabinosyl transferase
Fluoroquinolones	0.5–2.0 (7H9/7H10)	*gyrA/gyrB*	DNA gyrase
Kanamycin/amikacin	2–4 (7H9/7H10)	*rrs*	16S rRNA
Capreomycin/viomycin	2–4	*tlyA*	rRNA methyltransferase
Ethionamide	10 (7H11)	*inhA*	enoyl reductase
p-amino salicylic acid	0.5 (LJ)	*thyA*	thymidylate synthase A
PA-824 and OPC-67683	0.03 (7H9/7H10)	Rv3547	hypothetical 16.4 kDa
TMC207	0.03 (7H9/7H10)	*atpE*	ATP synthase

Table 2: Representing the antibiotics used for tuberculosis, with the MIC values, gene and gene products in *Mycobacterium* (Da Silva and Palomino, 2011).

1.6.1 Isoniazid

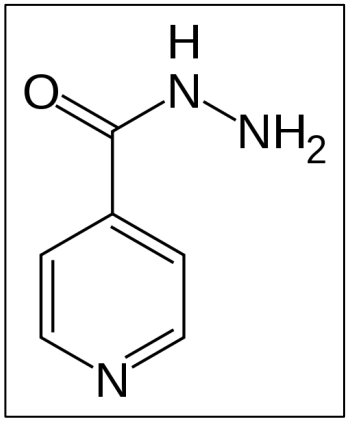


Figure 21: The chemical structure of Isoniazid

It is an antibiotic containing a pyridine ring and a hydrazide group (Fig.21) both of which are important components for its activity against *Mycobacterium*. Opposite to its simple structure its mechanism of actions is considered quite complex (Middlebrook et. al., 1953). It interferes with the synthesis of essential mycolic acids by inhibiting NADH-dependent enoyl ACP reductase that is encoded by a gene *ihnA*

in Mycobacterium (Rawat et. al., 2003). Strains that developed resistance against isoniazid have shown to have mutations within *inhA* and another gene called *katG*. (Silva et. al., 2003; Ramaswamy et. al., 2003). The mutation within *katG* known as S315T is a second step mutation caused due to inappropriate chemotherapy and is more frequently seen in MDR rather than isoniazid mono-resistant strains (Hazbon et. al., 2006). Mutation in *inhA* has been known to cause resistance in second line drugs as well due to the same mechanism of action (Banerjee et. al., 1994). Another gene called *ndh* when mutated decreases the activity of NADH dehydrogenase and therefore shows a resistance to both isoniazid and ethionamide (Miesel et. al., 1998).

1.6.2 Rifampicin

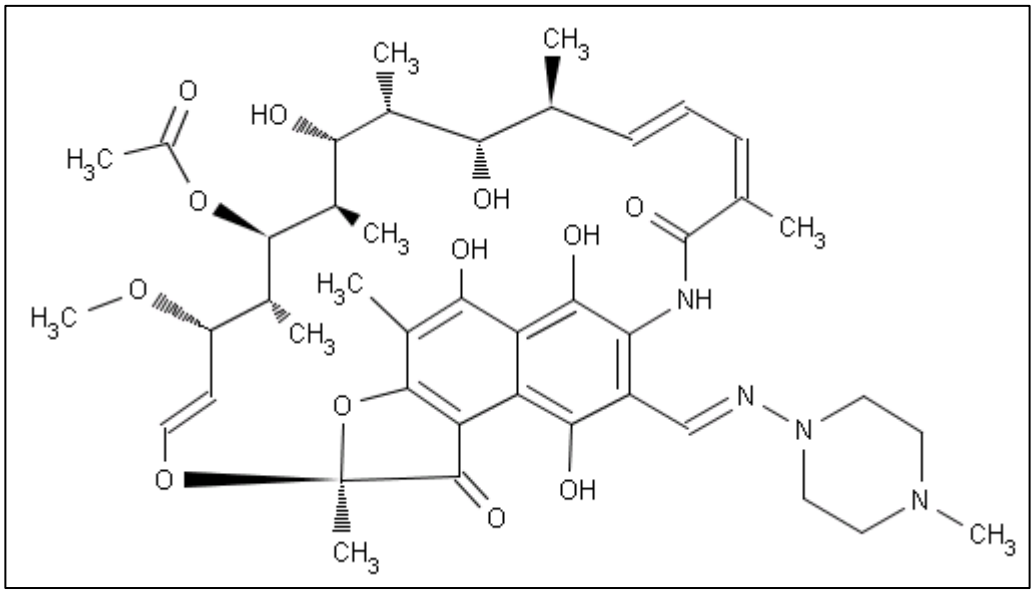


Figure 22: The chemical structure of Rifampicin

Rifampicin is a lipophilic ansamycin (Fig. 22) with antimicrobial activity (Rattan *et.*

al., 1998). It binds to the β- subunit of RNA polymerase and inhibits the elongation of messenger RNA (Blanchard, 1996). It acts against both active and non-growing bacilli. Resistance against rifampicin is generated by mutation with the *rpoB* gene in a region from 507-533 codon, called the rifampicin resistance-decreasing region (RRDR) (Ramaswamy *et. al.*, 1998). Resistance to rifampicin has shown the bacillus to be resistant to other drugs as particularly isoniazid (Traore *et. al.*, 2000).

1.6.3 Pyrazinamide

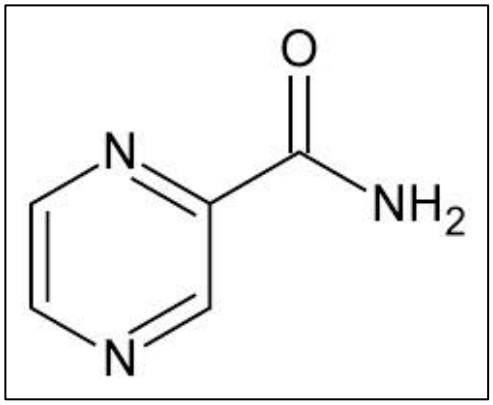


Figure 23: The chemical structure of Pyrazinamide

It is a structural analogue to nictoniamide (Fig. 23). It is a pro-drug, which is converted into its active form pyrazinoic acid via the enzyme pyrazinamidase or nicotinamidase (Pzase) (Konno et. al., 1967) encoded by pncA (Scorpio *et. al.*, 1996). The active form of the drug disrupts the bacterial membrane energetics and inhibits membrane transport. The drug is known to be able to inhibit semi-dormant bacteria living within acidic environment, which it does by disrupting the bacterial membrane having a low membrane potential (Zhang *et. al.*, 2003; Mitchison *et. al.*, 1985). It has also been suggested that pyrazinoic acid and n-propyl esters inhibit fatty acid synthase

type- I in replicating bacilli (Zimhony *et. al.*, 2007). Resistance against pyrazinamide is due to mutations that occur in the 561bp promoter open reading frame region in *pncA* gene (Scorpio et. al., 1997,Jureen et. al., 2008).

1.6.4 Streptomycin

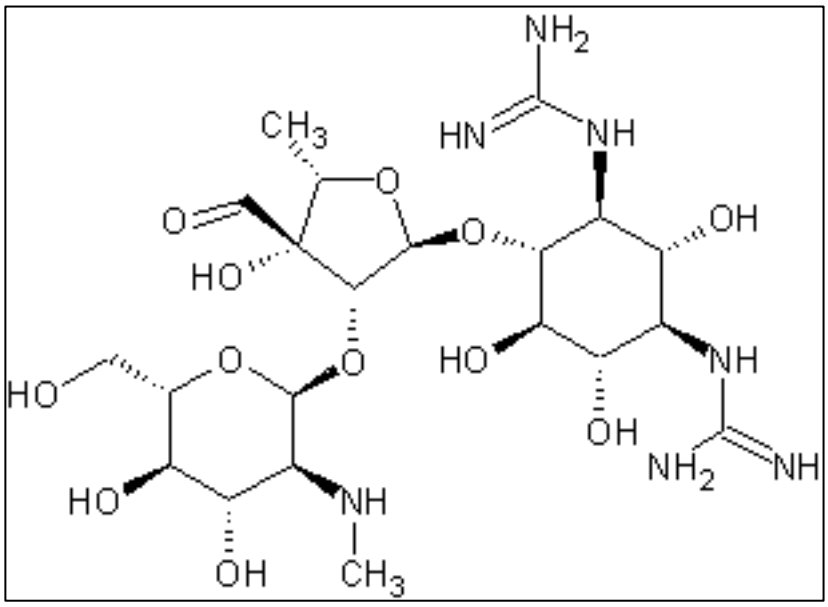


Figure 24: The chemical structure of Streptomycin

Streptomycin, an aminocyclitol glycoside antibiotic was the first antibiotic used for tuberculosis isolated from *Streptomyces griseus* (Fig 24). It inhibits mycobacterial growth by binding to the 16SrRNA (Moazed *et. al.*, 1987). Resistant strains emerged after its initial use as a monotherapy against tuberculosis (Crofton *et. al.*, 1948). Genetic resistance is responsible due to mutations in *rrs* and *rpsl* genes, which alter the streptomycin-binding site. Such mutations are observed in around 50% of strains (Gillespie et. al., 2002). The majority of point mutations are found in *rpsl*, most common being K43R. But their are resistant strains, which have been found containing no mutations in either of the two genes that suggest that there are other

genes, affecting resistance against streptomycin (Zhang *et. al.*, 2000).

1.6.5 Ethambutol

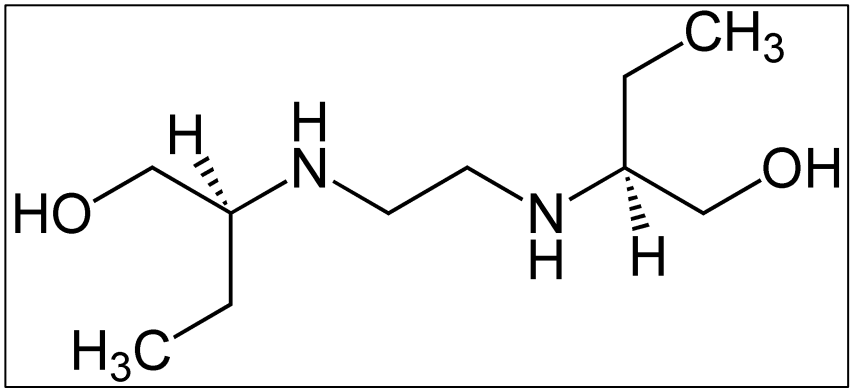


Figure 25: The chemical structure of Ethambutol

Ethambutol, having the chemical name (S, S) – 2,2' – (ethylenediimino) di- 1-butanol (Fig. 25) has been used as a first line drug in combination with isoniazid, streptomycin, rifampin and pyrazinamide to treat tuberculosis (Sreevatsan *et. al.*, 1997). The mode of action of the drug was said to be by inhibition of cell wall biosynthesis, mainly by two possible mechanisms of either direct inhibition of transfer of mycolic acid to the cell wall or by inhibiting synthesis of acceptors of mycolic acid (Takayama *et. al.*, 1979). In *M. smegmatis* the genes whose products were targeted by ethambutol were found to be in an operon, containing three genes *embA*, *embB* and *embC* (Sreevatsan *et. al.*, 1997). Recent development of resistance against antibiotics and study on mechanisms for antibiotic resistance lead to the discovery that mutations in specific codons in genes which are targeted by ethambutol cause resistance (Safi *et. al.*, 2008). Mutations in codon 306 in *embB* gene *(*Telenti *et. al.*, 1997) have been seen in most resistant strains and have been known to develop a resistance for other drugs mainly isoniazid and rifampin evolving into MDR (Safi *et.*

al., 2008).

1.6.6 p-Amino Salicylic acid

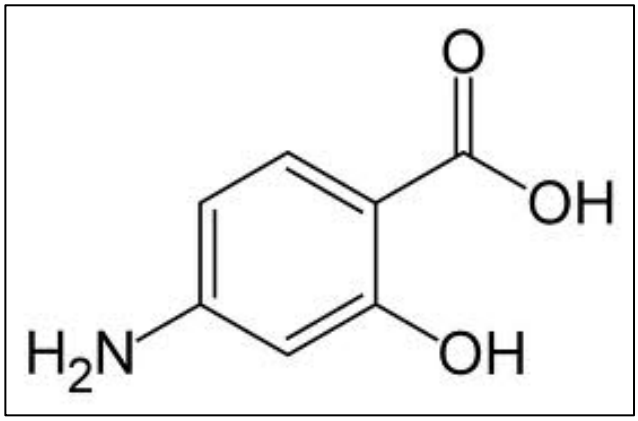


Figure 26: The chemical structure of p- Amino Salicylic acid

4- amino – 2- hydroxyl benzoic acid also known as p- amino salicylic acid (Fig. 26), was confirmed as a potent antitubercular drug against 12 virulent strains of human tubercule bacilli a few being resistant to streptomycin (James A. O' Connor, 1948). In the beginning the drug showed poor patient compliance due to gastro intestinal toxicity but after the development of a new formulation and a need for new antibiotics due to and increase in MDR cases the use of it as an antitubercular antibiotic was back in place. Due to its structural similarity to sulphonamides it was thought to be competing with PABA (p- amino benzoic acid) for dihydropteroate synthase (DHPS) in the folate synthesis cycle (Rengarajan *et. al.*, 2004). But when further investigated it was seen that PAS was a prodrug activated via a product from the gene *thyA* coding for thymidylate synthase A, an important enzyme in thymine synthesis (Rengarajan *et. al.*, 2004, Leung *et. al.*, 2010). PAS resistance is associated with mutations in the *thyA* gene, where in most common is the threonine to alanine conversion on the 202

position (Leung *et. al.*, 2010). Although this fact was challenged to a study proving the mutation to be a marker for Latin American Mediterranean (LAM) lineage of *M. tuberculosis* rather than cause for resistance to p-aminoslalicylic acid (Feuerriegel *et. al.*, 2010).

1.6.7 Ethionamide

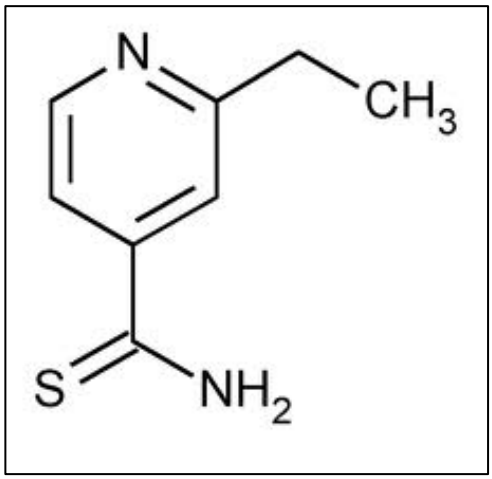


Figure 27: The chemical structure of Ethionamide

Ethionamide (Fig. 27) has a structure similar to isoniazid and is known to have a similar mode of action by inhibiting the mycolic acid biosynthesis. Studies show that both isoniazid and ethionamide target the gene *inhA* responsible for the synthesis of enoyl acyl ACP reductase in *Mycobacterium* (Wang *et. al.*, 2007). Mutation in the S94 allele of the *inhA* gene caused a five-fold increase in resistance against isoniazid and ethionamide in *M. tuberculosis* and *M. bovis BCG* (Vilcheze *et. al.*, 2006). Although it is proven that the target of both antibiotics might be the same the mode of activation for both is different. Ethionamide like isoniazid is a prodrug which is activated by the product of the gene *ethA* (Wang *et. al.*, 2007), responsible for

synthesis of monoxygenase (Leung *et. al.*, 2010) which activates the ethionamide by forming an adduct with NAD and inhibiting *inhA* (Vilcheze *et. al.*, 2008). 9 different types of mutations have been identified in *ethA* gene , which increases resistance to ethionamide by decreasing activation of the antibiotic (Leung *et. al.*, 2010). Another gene, which is a target of isoniazid, *katG* has been associated with ethionamide resistance. Mutation in the *katG* gene has been said to provide a favorable environment for the development of ethionamide resistance. (Hazbon *et. al.*, 2006). Some new genes such as *mshA* involved in mycothiol biosynthesis (Vilcheze *et. al.*, 2008) and *ndh* producing type II NADH dehydrogenase (Vilcheze *et. al.*, 2005) have been implicated in being involved in ethionamide function; as mutations in these genes have conferred resistance to *Mycobacterium* strains against both isoniazid and ethionamide,

The above mentioned antibiotic are used as treatment for tuberculosis but due to increases in strains of resistant *Mycobacterium* it is imperative to look for drugs with better efficacy and targets which will help improve the drug regimen for treatment of afflicted patients.

1.7 High Throughput Screening (HTS) for novel compounds with anti-tubercular activity

Screening of antibiotics was previously performed via various cell-based assays, which were manually curated and handled individually. With the development of assay plates or microwell plates containing wells ranging for 96 to 384 wells in a single rectangular plate; the process of screening was made simpler and quicker. But

with rapid increase in the number of synthesized antibiotics and compound libraries it became imperative to automate liquid handling systems and improvise assays to give quicker and accurate results with lesser discrepancies. The drawbacks of optical density based assays in terms of variation in cell number and growth rate lead to attempts at speeding up the process of drug discovery by using assays using redox dyes (alamar blue), tetrazolium for *Mycobacterium* to detect compound or antibiotic activity against the microbe (Singh *et. al.*, 2010). A compound library from NIH; SMR (small molecule repository) was screened using the Alamar blue end point assay to screen 215,110 compounds resulting in "hits" comprising of compounds ranging from substituted pyrimidines and quinolones to thiadiazoles and pyrazoles. (Maddry *et. al.*, 2009). Although, such assays seem to be depending on continuing C.F.U and long incubations with the dye as a main assessment technique which might lead to alteration of the metabolic and physiological state of the bacillus (Singh *et. al*., 2010). Even though whole cell screening assays have lead to new pathways and enzymes encouraging drug discovery (Sala and Hartkoorn, 2011), direct screening of *M. tuberculosis* is a challenging task as it involves the use of a BSL-3 containment with the HTS equipment. This has lead to the use of other *Mycobacterial* species as model organisms to be used for screening out drugs. Around 20,000 molecules from the Broad Institute were screened to get novel inhibitors targeting *DprE1* (enzyme for cell wall synthesis) and *MmpL3* (mycobacterial cell membrane protein large 3), which is an exporter of newly synthesized cell wall mycolic acids using GFP fluorescence as reporters (Stanley *et. al.*, 2012). HTS has also been used to validate inhibitors against specific targets such as *lspD* which is an enzyme used in the pathway for synthesizing isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) which play a role in the electron transport chain (Gao *et. al.*, 2012). Specific assays

against *M. tb* dihydrofolate reductase (important for folate pathway) involving the use of fluorescent dye resazurin, which measures the metabolic state of the cells was designed to screen out 32,000 synthetic and natural product derived compounds (Kumar *et. al.*, 2012) while a dual selection assay against 1-deoxy-D-xylulose-5-phosphate synthase (DxS) or 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DxR) was designed using an increase in NADH concentration to detect DxR inhibitors which could also screen out compounds against DxS simultaneously (Humnabadkar *et. al.*,2011). Novel HTS assays have been designed against non-replicating bacilli having antimicrobial tolerance by a low oxygen recovery assay using a plasmid containing acetamide promoter driving a bacterial luciferase gene adapted to low oxygen conditions. It was seen that agents against cell wall were inactive against NRP but have shown promise against other cellular targets (Cho *et. al.*, 2007). An assay against glutamine synthetase was designed, optimized and standardized to all conditions such as temperature, DMSO concentration and culture volume related to the assay to get a robust assay to screen 18,000 small molecules (Singh *et. al.*, 2005). An automated confocal based fluorescence microscopy HTS screening of 57,000 small molecules lead to 135 potent compounds such as dinitrobenzamide derivatives against the synthesis of LAM and AG synthesis including activity against XDR. (Christophe *et. al.*, 2009)

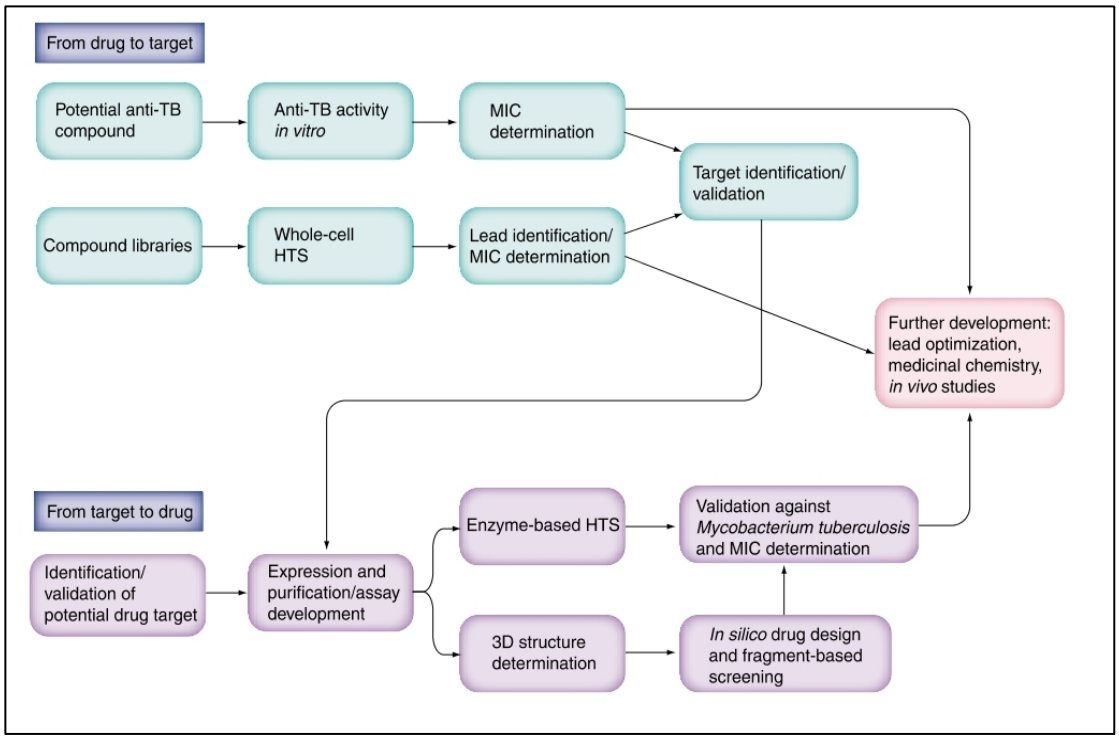


Figure 28: A flowchart representing drug discovery strategies describing the drug development from drug to target and from target to drug. The two strategies overlap at a level for in vivo studies. HTS – High throughput screening. (Sala and Hartkoorn, 2011)

There are two major ways in which screening of drugs seems to be carried out, either assays are designed to be specific against at target leading to inhibitors of specific enzymes, pathways or molecules whereas a more generalized approach involves cell based assays to reflect any inhibitory compounds which can be studied further for their mode of actions on the bacteria. The flowchart above summarizes the various strategies currently in place for drug discovery and development of antitubercular drugs using high throughput screening (Sala and Hartkoorn, 2011) (Fig. 28).

2. RATIONALE OF STUDY

Over the years the global scenario of tuberculosis has changed leading to worldwide emergency declared by the WHO in 1993. The control of which has been highly deterred due to the emergence of high number of drug resistant strains (Da silva and Palomino, 2011). The need of the hour therefore, points towards newer drug development and discoveries with increased speed and resilience. HTS of drugs and synthetic compounds has proved to be a useful and speedy platform for drug development. Although, a good assay for HTS should have a combination of a micro plate format, a low cost such as of reporter assays using fluorescent proteins or cell density measurement and redox dye assays, which use electron transport as an indicator (such as ATP, NADP, FAD conversions) with easy kinetic monitoring of the BACTEC system. (Collins et. al., 1998). A comparative analysis of BACTEC 460 assay and the Alamar Blue assay lead to the conclusion that as opposed to the expensive, cumbersome and radioactive assay the alamar blue assay is a reliable, non-radioactive and cheap assay for HTS (Collins and Franzblau, 1997). The use of redox dyes as such does lead to questionable physiological changes that the bacteria might go through during incubation (Singh et. al., 2010) and also its difficult nature for kinetic or real time monitoring of antibiotic activity (Collins et. al., 1998). Fluorescence assays can prove to be better kinetic as well as end point assays as seen from many drug screens utilizing fluorescent dyes such as resazurin (Kumar et. al., 2012) and green fluorescence proteins (Christophe et. al.2009).

Therefore an optimized and validated assay utilizing fluorescent proteins produced by

the bacillus could give real time results of antitubercular activity as well as decreasing the risk of interaction with other dyes and reagents at the same time, which would not influence the physiology of the bacillus.

3 AIM AND OBJECTIVES

3.1 Aim: Development of a phenotypic HTS assay to identify novel molecules that inhibit *Mycobacteria*.

3.2 Objectives:

1. To create genetically modified strains of *M. smegmatis* expressing fluorescent proteins for assay development.

2. To optimize conditions such as media, cell concentration, time range, antibiotic concentration etc. required to get a reliable and repetitive assay.

3. To screen compounds and drugs from libraries against the *Mycobacterium* strain for detection of antimycobacterial activity.

4. MATERIALS AND METHODS

4.1 Antibiotic stocks

Antibiotics were prepared as stock solutions at a higher concentration to be diluted to different working concentrations as per requirement.

4.1.1 Standard antibiotics

Requirements: For 10mM stocks (5mL) – Isoniazid – 6.85mg, Ethambutol – 13.86mg, Streptomycin – 36.44mg, Pyrazinamide – 6.16mg, Rifampicin – 41.15mg

For 100mg/mL (5mL) – All antibiotics required at 500mg.

Procedure: The standard antibiotics (Isoniazid, Ethambutol, Rifampicin, Pyrazinamide used were prepared as stocks in DMSO, while Streptomycin was prepared in sterile water as solutions at concentrations of 10mM and 100mg/mL. The antibiotics were filter sterilized using a 0.2µm pore size filter.

4.1.2 FDA Drugs

Requirements: For 10mM stocks (250µL) – Tamoxifen citrate – 1.4091mg, Meclocycline sulfosalicylate salt – 1.737mg, Clomiphene citrate salt – 1.4952mg, GBR 12909 dihydrochloride – 1.308mg, Sulocitidil – 0.8439mg, Fendiline hydrochloride – 0.8797mg, R- (-)- Apomorphine hydrochloride hemihydrate – 0.782mg

Procedure: The FDA drugs (Tamoxifen citrate, Meclocylcine sulfosalicylate salt, Clomiphene citrate salt, GBR 12909 dihydrochloride, Sulocitidil, Fendiline hydrochloride, R- (-)- Apomorphine hydrochloride hemihydrate) selected after the screen were prepared as stocks at concentrations of 10mM in DMSO.

4.1.3 Stock antibiotics for screening transformed M. smegmatis

Requirements: For Kanamycin stocks 10mL – 500mg; Hygromycin stock (available)

Procedure: Kanamycin was prepared as a stock solution of 50mg/mL in water and was filter sterilized using a 0.2µm pore size filter. The working concentration of Kanamycin was 10µg/mL.

Hygromycin was available as a stock solution in PBS at a concentration of 50mg/mL and was used at a working concentration of 20 µg/mL

4.2 Culture media

Various culture medias were used to grow *Mycobacterium smegmatis* to ensure optimum growth conditions for a reliable assay.

4.2.2 Tryptic Soy Broth (TSB)

The TSB media was prepared by dissolving 7g of TSB in 250mL of distilled water. The solution was then autoclaved at 121°C for 20 minutes.

4.2.3 Tryptic Soy Agar (TSA)

The TSB agar was prepared by dissolving 7g of TSB, 3g of Agar in 250mL of distilled water. The solution was then autoclaved at 121°C for 20 minutes. The solution was brought to around 50-60 °C then poured into sterile petri plates and allowed to solidify.

4.2.4 Tripticase Soy Broth

The TSB media was prepared by dissolving 7.5 g of Tripticase SB in 250mL of distilled water. The solution was then autoclaved at 121°C for 20 minutes.

4.2.5 Middlebrook 7H9 Broth

The 7H9 broth was prepared by dissolving 1.31g of Middlebrooke 7H9 in 250mL of distilled water. To which 556µL of Glycerol was added. The solution was then autoclaved at 121°C for 20 minutes.

4.2.6 Tween- 80 (10%)

Polysorbate 80 or Tween 80 is a hydrophilic non-ionic surfactant and emulsifier used in the culturing of *Mycobacterium* sps., to avoid clumping of cells and get an even

suspension of cells. A 10% tween-80 solution was prepared by dilution in distilled water followed by filter sterilization using a 0.2µm pore size filter.

4.3 Culturing bacterial cells

4.3.1 Wild type M. smegmatis (mc^2 155)

Small-scale cultures were setup in 20mL culture bottles with 10mL TSB, 50µL Tween 80 (10%). A loopful of colony from a streaked plate was inoculated in the medium and incubated at 37°C, 150rpm, 48hrs.

Scale up cultures were then setup using the small scale culture batch as inoculum with 100mL TSB, 50 µL Tween 80 (10%) and 2mL of inoculum at 37°C, 150rpm, overnight.

4.3.2 M. smegmatis (pMind_gfp)

The plasmid used to transform was pMIND_gfp, which was a plasmid whose gfp expression was regulated by the conditional presence of tetracycline. It was derived from a plasmid containing the *lux*AB gene encoding the luciferase enzyme. The plasmid has a gfp codon alongwith an antibiotic marker for kanamycin. The expression of gfp is controlled by a tetracycline stimulation (Fig. 29) (Blokpoel *et. al.*, 2005).

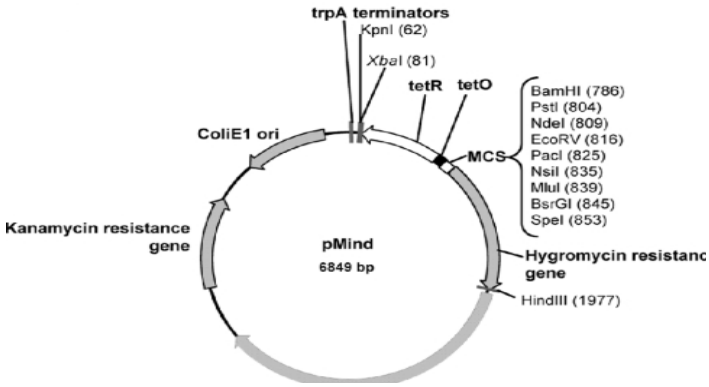


Fig 29: pMind_gfp plasmid with a Kanamycin resistance gene and the tetracycline gene for controlled production of GFP in the cell (Blokpoel *et. al.*,2005)

Small-scale cultures were setup in 20mL culture bottles with 10mL TSB, 50µL Tween 80 (10%), 10µL Kanamycin (50mg/mL). A loopful of colony from a streaked plate was inoculated in the medium and incubated at 37°C, 150rpm, 48hrs.

Scale up cultures were then setup using the small scale culture batch as inoculum with 100mL TSB, 50 µL Tween 80 (10%), 100µL Kanamycin (50mg/mL) and 2mL of inoculum at 37°C, 150rpm, overnight.

4.3.4 M. smegmatis (pSMT3_egfp)

The plasmid used to transform the strain was pSMT3_egfp, which has a shuttle vector pSMT3 for Mycobacterium with an hsp60 promoter (Hayward *et. al.*, 1999). It has restrictions sites for BamH1 and Hind III digestion sites. It has been cloned with an enhanced GFP codon and has an antibiotic marker for hygromycin. The GFP expression is independent of the hygromycin induction (Fig.30) (Humphreys *et. al.*, 2006; Abdallah *et. al.*, 2006).

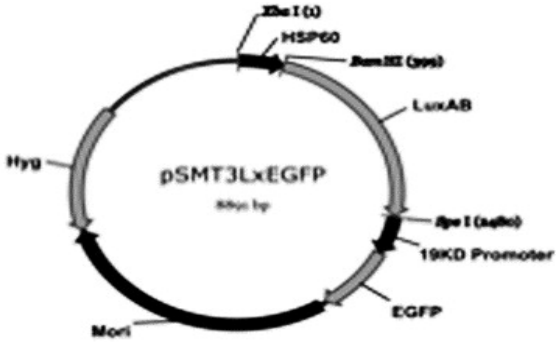


Fig 30 : The pSMT3_sgfp plasmid with a Mycobacteria shuttle vector pSMT3; hygromycin marker, and enhanced GFP codon with a hsp60 promoter (Humphreys *et. al.*, 2006)

Small-scale cultures were setup in 20mL culture bottles with 10mL TSB, 50µL Tween 80 (10%), 20µL Hygromycin (50mg/mL). A loopful of colony from a streaked plate was inoculated in the medium at incubated at 37°C, 150rpm, 48hrs.

Scale up cultures were then setup using the small scale culture batch as inoculum with 100mL TSB, 50 µL Tween 80 (10%), 200µL Hygromycin (50mg/mL) and 2mL of inoculum at 37°C, 150rpm, overnight.

4.4 Transformation of *M. smegmatis* wild type strain*(mc^2 155)* to GFP strains

To produce genetically modified strains of *M. smegmatis* expressing green fluorescence protein for HTS screening of drugs, the plasmids with a GFP marker had to be electroporated into wild type *M. smegmatis* strains. The procedure involved

producing electrocompetent cells to electroporate the said plasmids and then screening out the transformed cells.

4.4.1 Preparation of electrocompetent cells

Requirements: 10% sterile glycerol, Ice, wild type *M. smegmatis* (100mL, $O.D_{600}$ - 0.8)

Procedure: 100mL large-scale culture of wild type *M.smegmatis* was cultured overnight. The culture was then incubated on ice for 1.5hrs after which it was centrifuged at 3000g, 10 minutes. The cells were washed with ice cold 10% glycerol 3 times reducing the volume each time. A 100 mL culture after centrifugation was washed with 25mL; 10mL and 5mL of 10% ice cold glycerol consecutively. The electrocompetent cells were then aliquoted and stored at -80°C after flash freezing with liquid nitrogen (-196°C).

4.4.2 Electroporation of electrocopetent cells with GFP gene containing plasmids

Requirements: Electrocompetent cells, electroporation cuvets (0.2cm gap electrodes), electroporation apparatus with pulse controller, Plasmid DNA in solution (approx. concentration 0.2-1mg/mL), TSB, Ice.

Procedure: 5µL of plasmid DNA was added to 200 µL of electrocompetent cells and kept in ice for 10minutes. The cells were then transferred into an ice-cold sterile electroporation cuvet ensuring that there are no bubbles in the culture in the cuvet.

The cuvet was then placed in the electroporation chamber and subjected to a single pulse of 1.8kV with pulse controller resistance set at 1000Ω resistance. The cuvet was pot back in ice for 10minute. The suspension was transferred to a sterile microcentrifuge tube (1.5mL) and 5mL of TSB was added to it. The cells were then incubated at 37°C for 4hrs. The cells were then plated on agar plates containing selective antibiotic for each plasmid and incubated at 37°C for 3-7 days till colonies appear.

Plasmids transformed into wild type *M. smegmatis* were pMind_gfp (Kanamycin resistant marker) and pSMT3_egfp (hygromycin resistant marker).

4.4.3 Glycerol Stocks

Glycerol stocks were prepared using fresh cultures with an $O.D_{600}$ of 0.5-0.6. The stocks were prepared by adding 1mL of bacterial culture, 357 µL of sterile glycerol (70%) so that the final concentration of glycerol reaches 25% in the culture. The cryovials were then flash frozen using liquid nitrogen (-196°C) and then stored at -80°C.

4.5 Assay Optimization

The assay runs started with growth curve analysis using standard antibiotics used for M. tuberculosis. The runs were carried out on wild type *M. smegmatis* (positive control) and the transformed *M. smegmatis* strains. The assays run were tweaked and corrected from changing media, maintaining optimal DMSO concentrations, deciding optimal time range and finalizing the GFP strain to achieve the best possible assay

conditions suitable to run a compound or drug screen. The drugs were screened through HTS by using the automated MICROLAB® STAR Liquid Handling Workstations by Hamilton. Plate preparation was manual or automated depending on the assay specifications. Assay plate creation methods were used to create intermediate plates, plates for assays and setup up read time intervals.

4.5.1 Assay Run 1

The assay setup was done beginning with validating any innate resistance in the *M. smegmatis* (mc^2155) strain against the antibiotics used in screening the transformed bacterial strains. The assay plates were setup with varying conditions to rule out possible loopholes for error. The assay plate setup for the preliminary run with transformed *M. smegamtis* was done to check the effectiveness of strains as screening indicators. For *M. smegmatis* (pMind_gfp) conditional presence of tetracycline was required to induce gfp production while for M. smegmatis (pSMT3_egfp) gfp was constitutively produced. In order to use the minimum concentration of tetracycline to rule out its effect on bacterial growth during final screening various concentrations of tetracycline were tested to observe a good level of fluorescence.

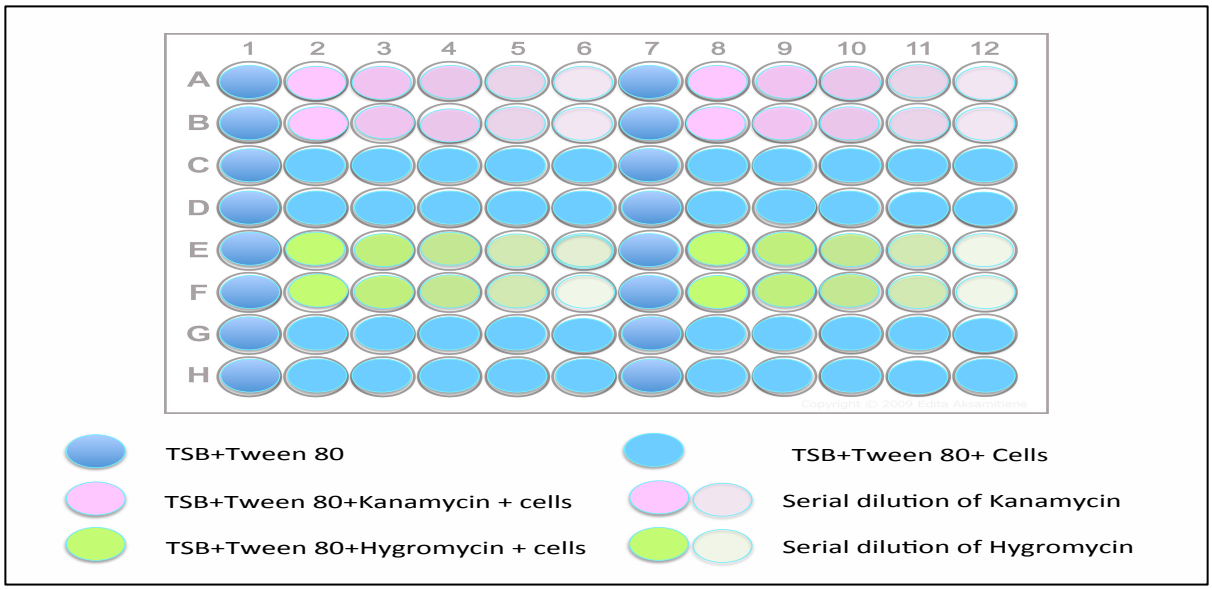


Figure 31: The above assay plates depicts the setup for a positive control plate with wild type *M. smegamtis* (mc^2 155) in the presence and absence of both hygromycin and kanamycin; to check for innate resistance.

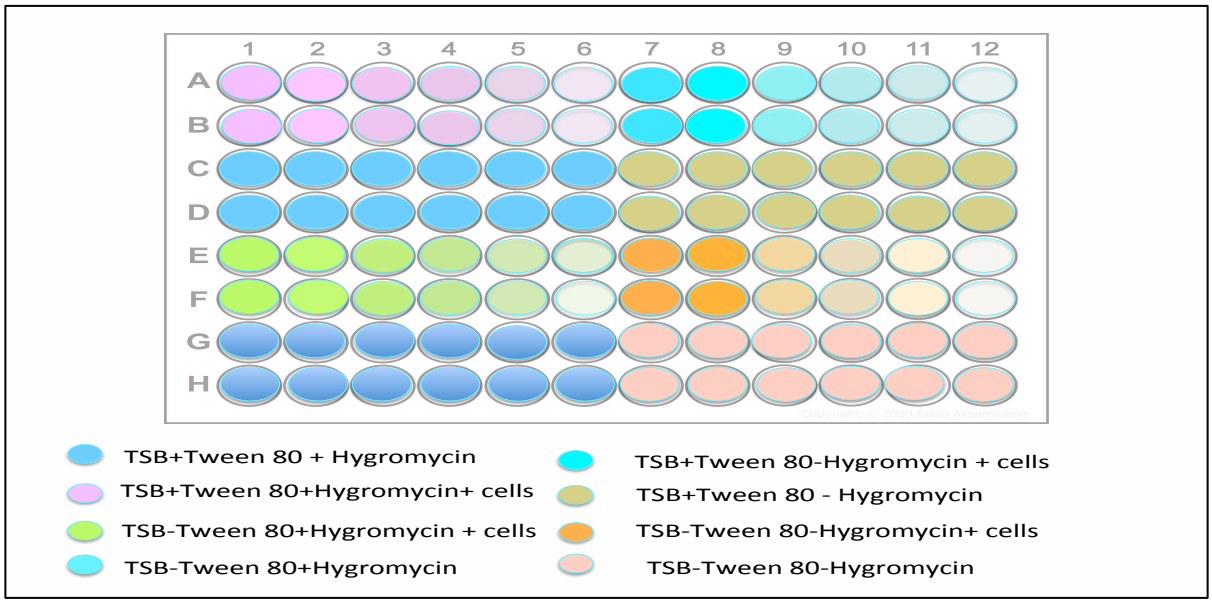


Figure 32: The above assay plates depict the setup for *M. smegmatis* (pSMT3_egfp) with varying conditions; presence and absence of Tween 80 and hygromycin.

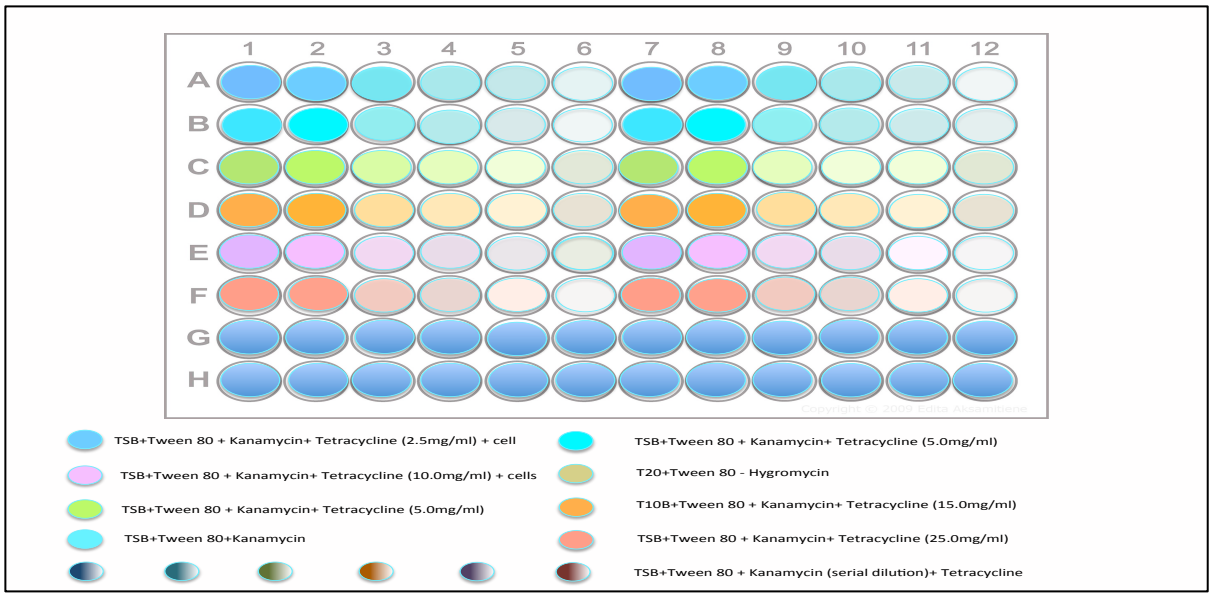


Figure 33: The above assay plates depicts the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pMind_gfp) with varying conditions; presence of Kanamycin and tetracycline. Two plates were setup in the same manner with different strains to have half of the plate as a duplicate for the experimental validation.

4.5.2 Assay Run 2

After observation of the fact that there was no innate resistance of *M. smegmatis* (mc^2155) against the antibiotics kanamycin and hygromycin another assay was done to get appropriate fluorescence window to get a reasonable cut off value. This was done by varying the cell concentration in two ways by diluting it in an equal ratio with the medium and by serially diluting the cells to 10 folds. Observing the fluorescence levels in the presence of various tetracycline concentrations, we decided on narrowing down the concentrations to three main concentrations that were 5.0ng/mL, 10.0ng/mL and 15.0ng/mL. Also to check for any background fluorescence observed media controls were maintained both with and without tetracycline. Cells were again used at 1:1 dilution with the media and 10 fold serial dilution; although the

media was changed from TSB to 7H9 as TSB and other similar substitute medias such as Tripticase SB showed high level of internal fluorescence causing low assay quality and high error.

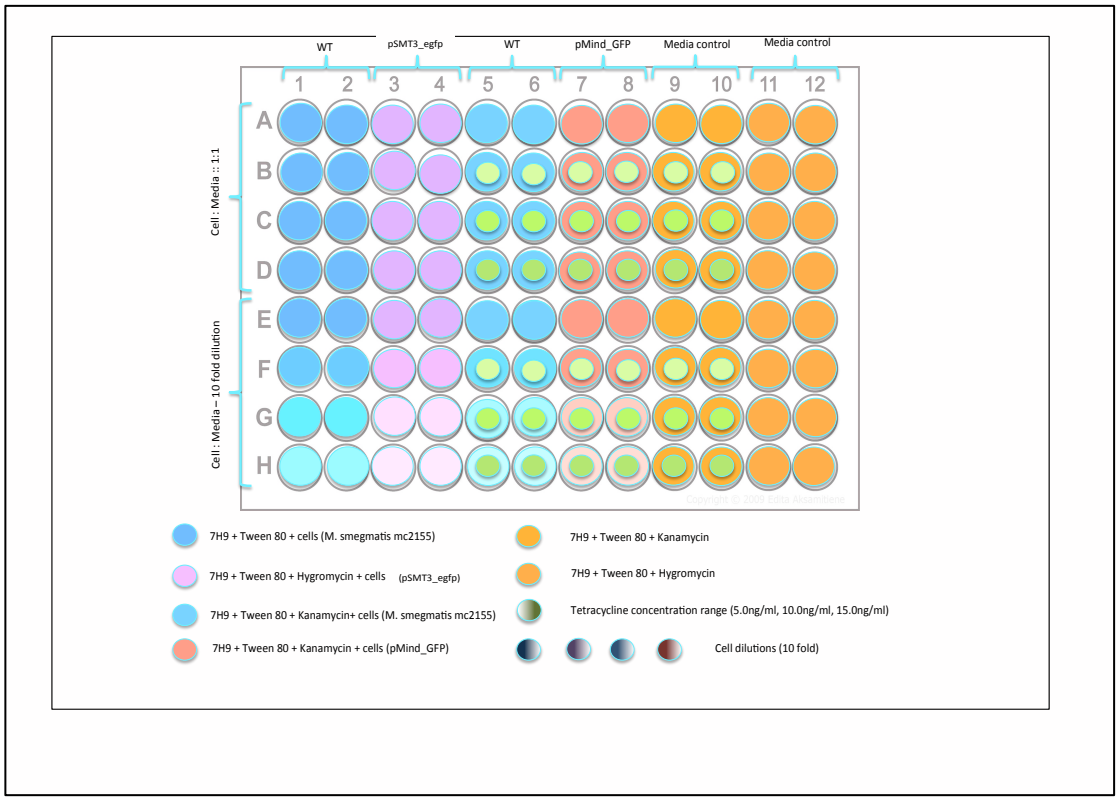


Figure 34: The above assay plates depicts the setup for *M. smegmatis* (mc^2155), *M. smegmatis* (pSMB_egfp) and *M. smegmatis* (pMind_gfp) with varying conditions; presence of Kanamycin and tetracycline. All conditions were performed in duplicate rows to maintain assay validation.

4.5.3 Assay Run 3

Observing background fluorescence from tetracycline and irregular fluorescence values; we finally selected *M. smegmatis* (pSMT3_egfp) as an indicative strain to do further drug screenings. It also seemed that cell counts when diluted in an equal ration

gave better values rather than serially diluted cells. Therefore, two plates with 2 fold serially diluted antibiotics (10mM stock concentrations) were prepared with two strains of *M. smegmatis* (mc^{2}155 and pSMT3_egfp) alongwith media controls. The test was once again done in duplicate sets.

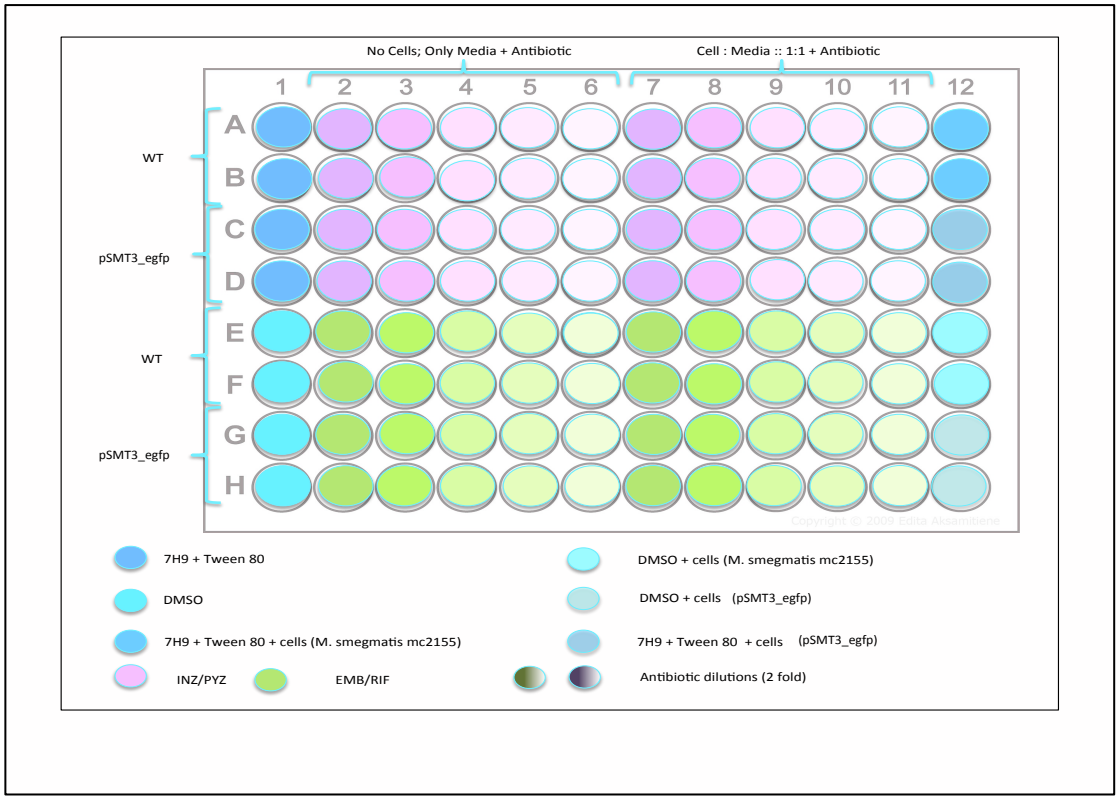


Figure 35: The above assay plates depict the setup for *M. smegmatis* (mc^{2}155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

4.5.4 Assay Run 4

Since the antibiotics were prepared in DMSO, which has shown to inhibit *M. smegmatis* growth DMSO controls was run alongwith the next assay. Also the number of antibiotic dilutions in the previous assay did not show much variation in growth curve as based on antibiotic concentration; therefore the number of dilutions were increased for the next assay run. Two plates were individually setup to run an assay on all four antibiotics.

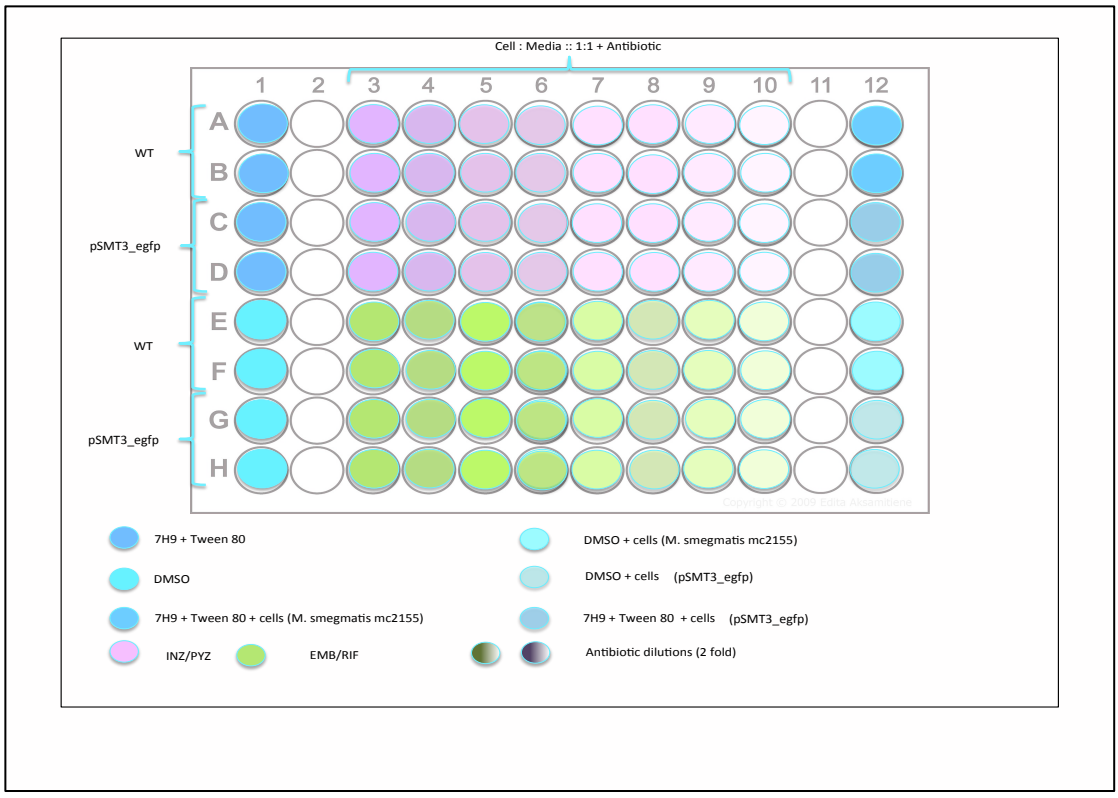


Figure 36: The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds and DMSO controls alongwith media controls. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

4.5.5 Assay Run 5

A repeat assay from the above run with slight variation in terms of checking different DMSO concentrations and its effect on *M. smegmatis* both wild type and pSMT3_egfp-containing strains was performed. The DMSO concentrations were varied as 1.25%, 2.5% and 5%.

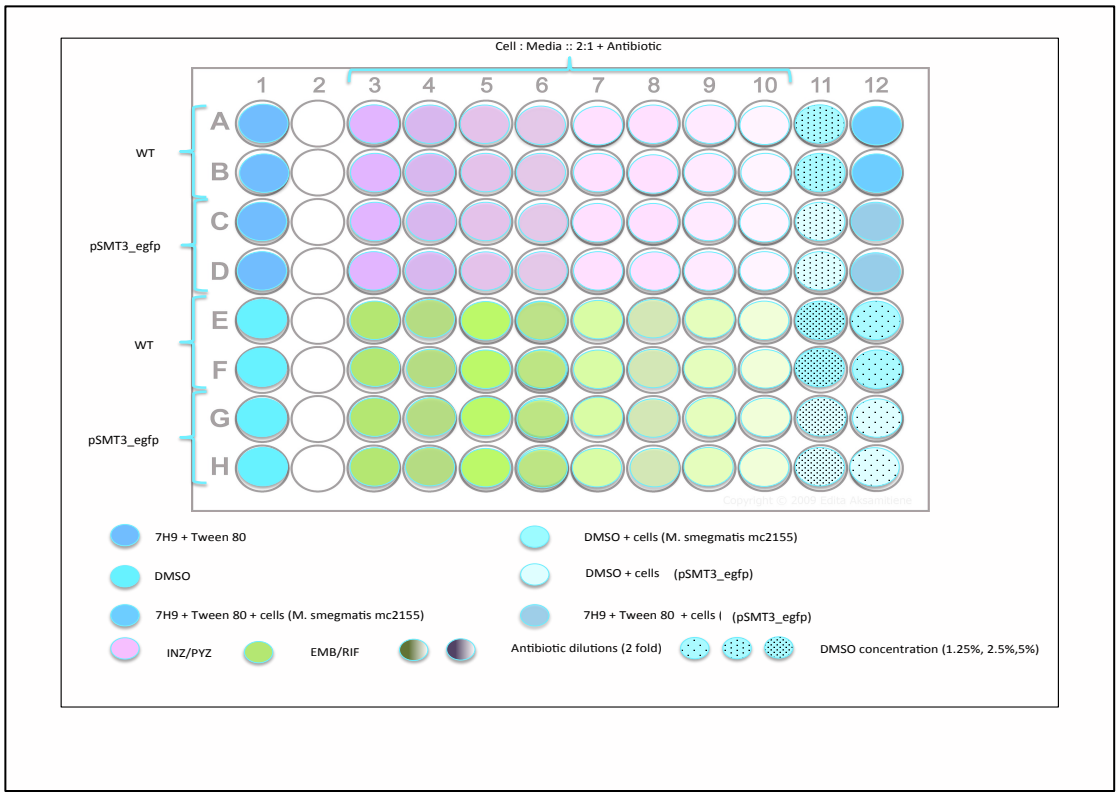


Figure 37: The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds and DMSO controls (1.25%, 2.5%, 5%) alongwith media controls. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

4.5.6 Assay Run 6

The screen for the compound library was finally setup using and intermediate plate prepared from the stock plates. The stock plates had the compound concentration at a 100mM and were diluted 90: 10 (90µL sterile water and 10µL antibiotic from the stock plate). Therefore the concentration of antibiotic in the intermediate plate was 10mM while the concentration of DMSO was decreased to 10%. Further when the antibiotics are added to the assay plate at a volume of 10µL in 200µL cell volume (100 µL of cells diluted as 1:1:: media: culture and 100 µL media; making the cells diluted as 2:1::media: cell); the DMSO concentration goes down to 0.5% and the antibiotic concentration in the final screen is at 0.5mM. The drug screen was run for a compound library with ID numbers RW036141- RW036146 and an FDA approved drug library from ID numbers RN000205 to RN000216; every plate containing 80 compounds or drugs respectively. Using the HTS robotic screening with the developed assay 480 compounds and 960 FDA approved drugs were screened against *M. smegmatis* for presence of any anti-tubercular activity. Alongwith the library assay plates, standard antibiotic plates were run to check effective working of the designed assay. Two identical plates were run for every screen including the antibiotic standards. The plates were read at every 3 hours over a 24-hour period and incubated in a static incubator.

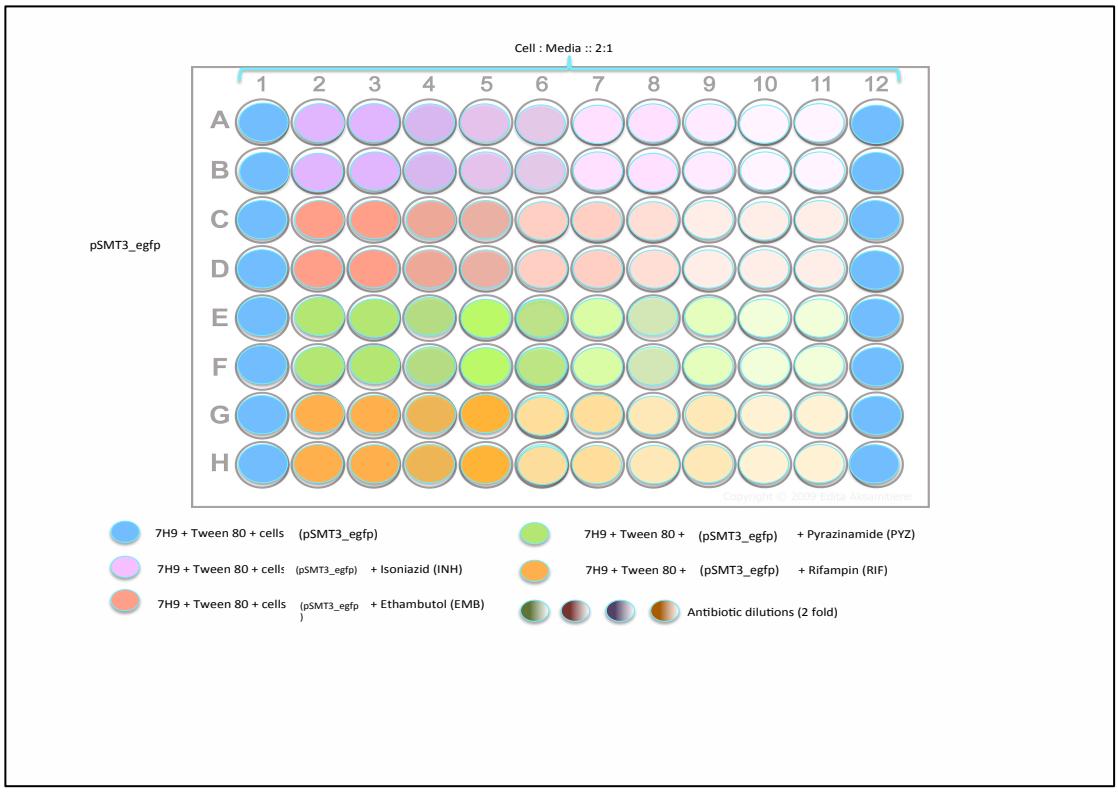


Figure 38: The above assay plates depict the setup for *M. smegmatis* (mc^{2}155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

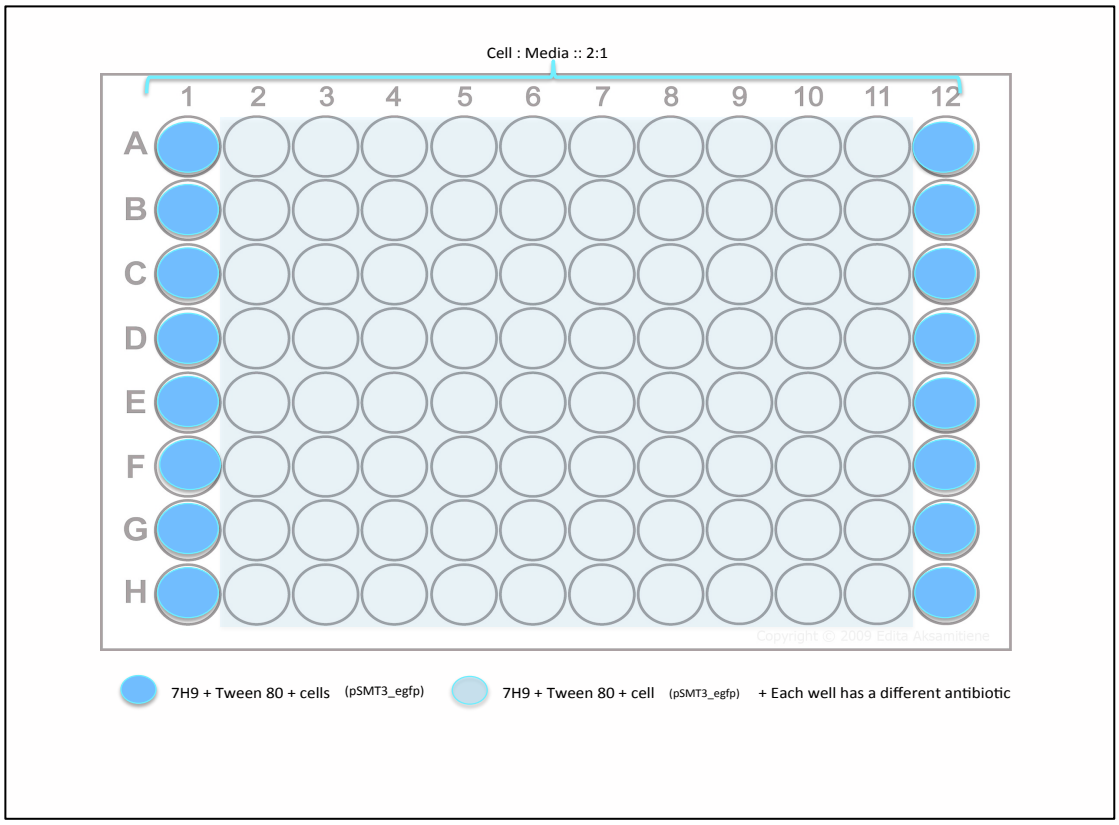


Figure 39: The above assay plates depict the setup for screening library compounds and drugs with the columns 1 and 12 as positive controls while the columns 2-11 had different compounds/drugs in each well.

4.5.7 Assay Run 7

The FDA drug library was run in two batches stock plates. The screen, which was run for plate ID RN000205 - RN000210, gave several hits representing novel inhibitory activity against *M. smegmatis*. The compounds were then used to do a dose response study to determine the minimum inhibitory concentration (M.I.C) for that particular drug. The initial concentration used during screening was 0.5mM; which showed a growth inhibition at or below 50%; therefore deeming the drug as being effective. For the dose response study concentrations below that value were taken starting at a 3-

fold dilution of 0.5mM; which presented concentrations as 0.166666667mM as the maximum concentration and moving to ten 3-fold dilutions of the same from well 2 to 11. The first column having a positive control (*M.smegmatis* without antibiotic) and column 12 having a negative control (only media).

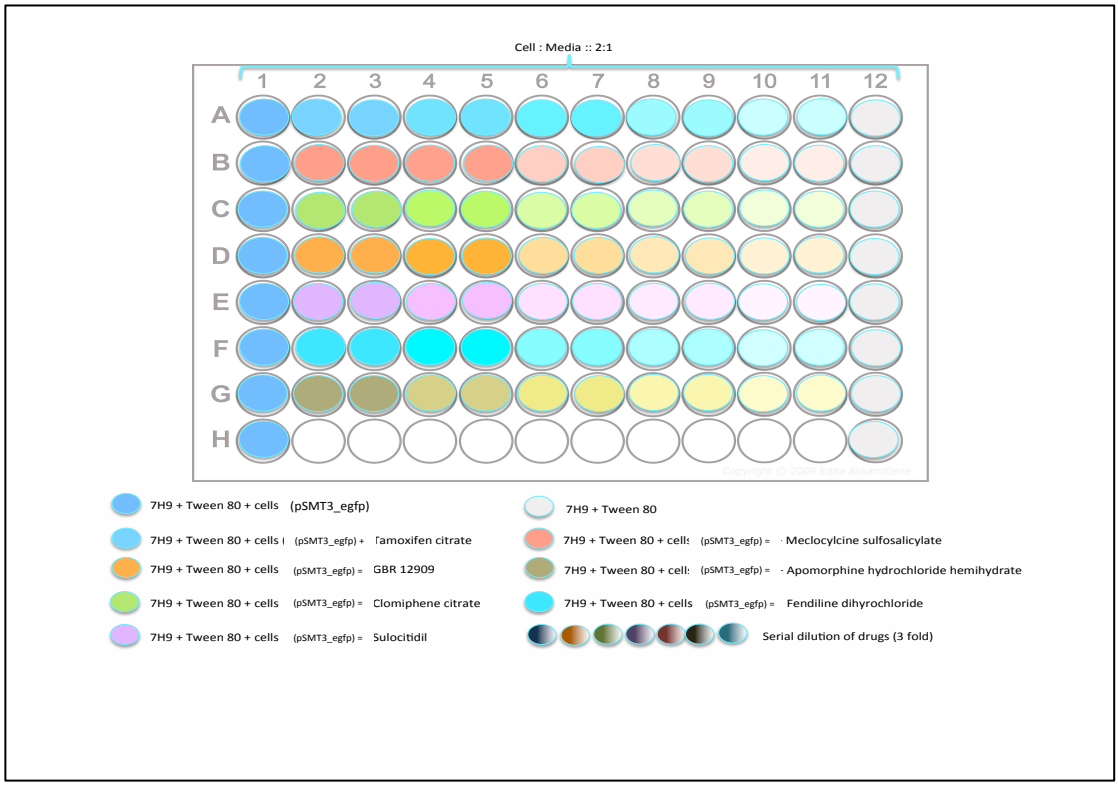


Figure 40: The above assay plates depict the setup for a dose response study of selected FDA approved drugs (A-G) serially diluted (3-fold) from 2-11; with column 1 as a positive control and column 12 as a negative control. A. Tamoxifen citrate, B. Meclocycline sulfosalicylate, C. Clomiphene citrate salt, D. GBR 12909 dihydrochloride, E. Sulocitidil, F. Fendiline hydrochloride, G. R- (-)- Apomorphine hydrochloride hemihydrate

The values attained from the above mentioned assays in terms of relative fluorescence units over a period of time depending on the number of readings and incubation

period was used to attain growth curves, survival percentages and percentage activity of the drugs in variation with doses.

4.6 Statistical Validation of Assays

All assays which were run were statistically validated for their quality using Z' factor values. The Z'factor mainly depicts the total separation between values of both positive and negative controls and the error within each control; describing the available signal window. The formula for calculating the Z' factor values is

$$Z` = 1 - \left(\frac{3\sigma_{+c} + 3\sigma_{-c}}{| \mu_{-c} - \mu_{+c} |} \right)$$

where σ_{+c}, σ_{-c}, μ_{+c} and μ_{-c} are the standard deviation (σ) and the averages (μ) of the positive (+c) and negative (-c) controls.

The values of Z' factor depicts how good or bad an assay is in terms of its reliability. In order for an assay to be ready for screening, the Z` should be at the very least 0.5, and much more preferably $\geq$ 0.6. If the Z` is <0.6, the assay quality is not good or reliable. A Z' factor value of 1 is considered as idle. (Zhang et. al., 1999)

5. RESULTS

5.1 Assay Optimisation

The primary experiment was designed to test which plasmid was most suited for performing antimycobacterial activity screens for *M. smegmatis.* The experimental conditions and related results as shown in Fig. 41, presented *M. smegmatis* (pSMT3_egfp) as a better candidate to proceed as a strain for the screening assay. The major reason being that the GFP production was not conditional to the selective antibiotic reducing the chance of the antibiotic affecting the screening results. The pMind_gfp strain had a conditional production in the presence of tetracycline. Also the results from the above assay run revealed that the medium used for growing the culture and diluting the cells that is tryptic soy broth (TSB) and trypticase soy broth was affecting the level of fluorescence for unknown reasons. Therefore for the purpose of a good screen the medium was shifted to a clear 7H9 broth.

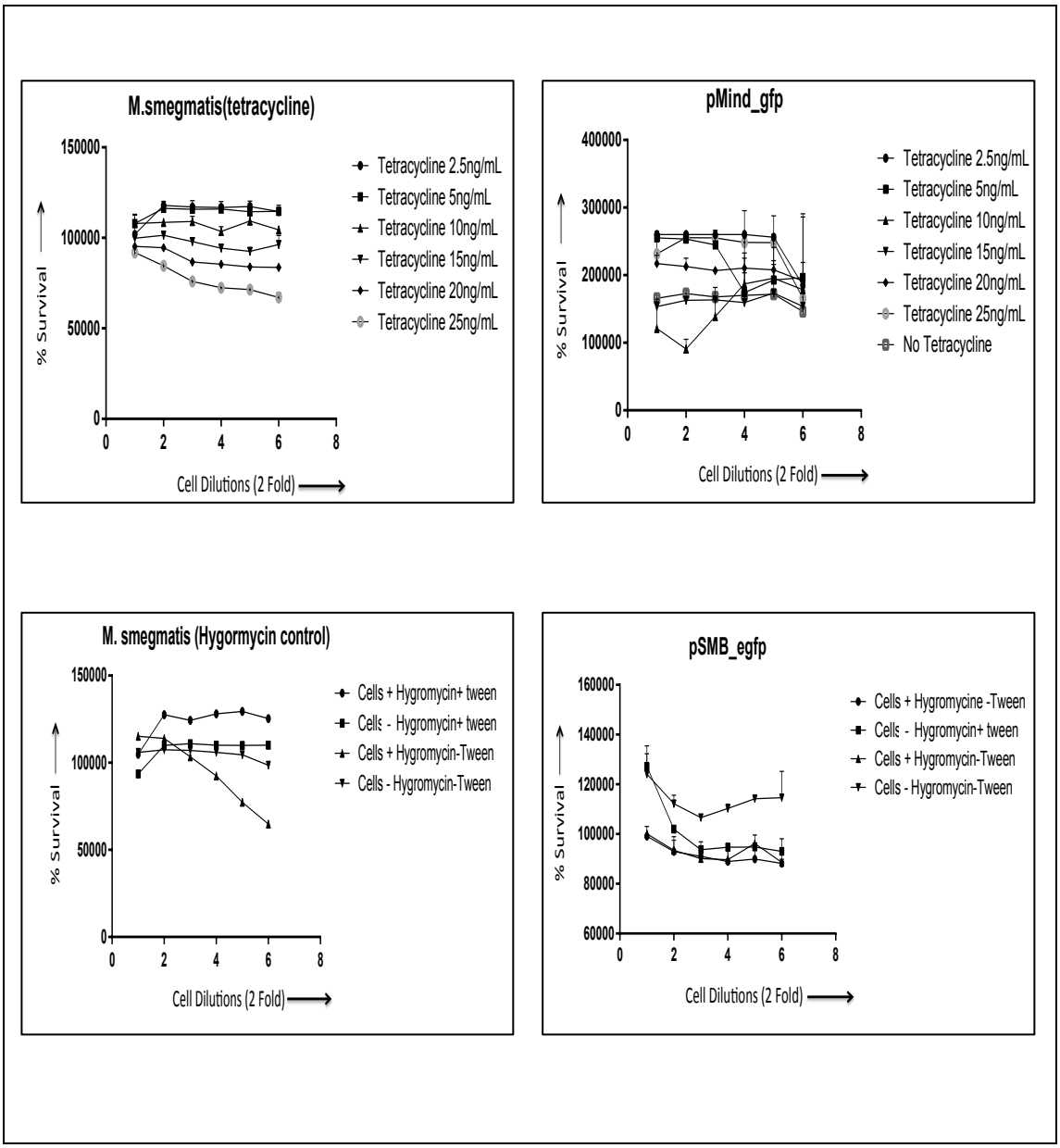


Figure 41: Graphs representing survival percentages of *M. smegmatis* (mc^2155), pSMB_egfp and pMind_gfp. Survival curves for pMind_gfp are represented for varying concentration of tetracycline (2.5ng/ml – 25ng/mL) with an M. smegmatis (mc^2155) control. Survival curves for pSMT3_egfp represent varying conditions of hygromycin and Tween-80 with M. smegmatis (mc^2155) as a control.

5.2 Assay Standardisation

After the determination of a model strain to be used for the screens, assays were setup using standard drugs to determine cell volume, concentration and drug volume, which would give effective results. The graphs in Fig 42 represent the cell dilutions, which were standardized against standard drugs that are Isoniazid, Ethambutol, Pyrazinamide and Rifampicin. The cells at 1:1 dilutions although showed a better response and values for fluorescence than undiluted cell, it quickly became clear that due to high cell load the fluorescence levels were quickly saturated after several cycles leading to inconclusive results (Fig. 42a.). When the cells were further diluted to a 2:1, media to cells ratio it was seen that there was a much clearer difference in cell survival percentage values versus antibiotic concentration. Therefore for the purpose of further assay development the cell concentrations were taken at a 2:1 dilution (Fig. 42 b.). The drug standards run alongwith the compound screens also showed clear survival curves validating the effectiveness of the changes made to the assay.

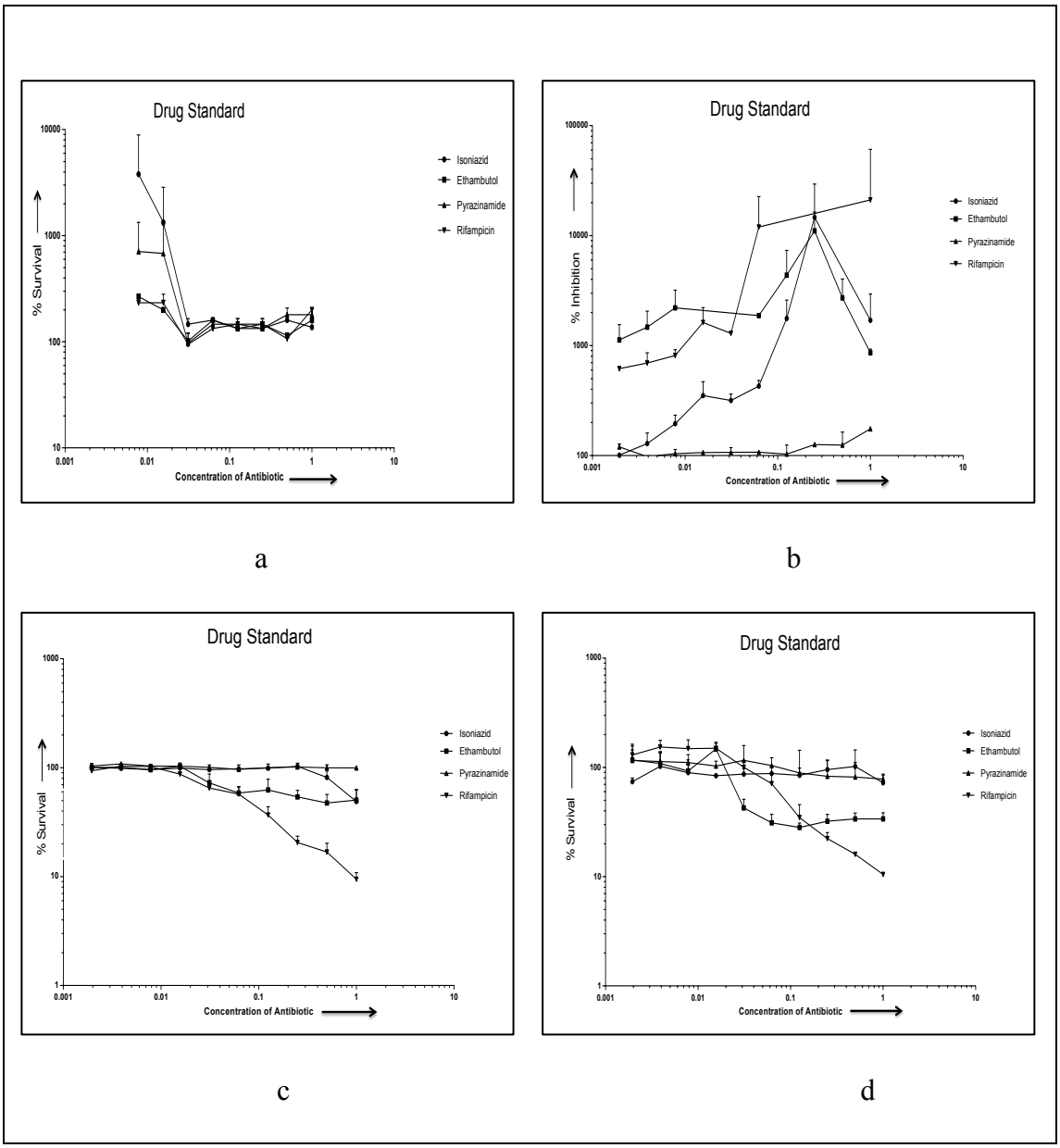


Figure 42: Graphs representing survival and inhibition percentages of M. smegmatis (pSMT3_eGFP) against standard drugs like Isoniazid, Ethambutol, Pyrazinamide and Rifampicin over dilutions of 2 folds. a. and b. Representing the survival percentages during early optimization of cell concentration (1:1 and 2:1 respectively). c. and d. represent the drug standards run during the compound library screening. Z' factor – 0.6-0.7

5.3 HTS screen against diversity library

The compound screen resulted in many "Hits", which were considered to be all those compounds, which caused the survival percentage of *M. smegmatis* (pSMT3_egfp) to be at and below 50% value as depicted in Fig. 43.

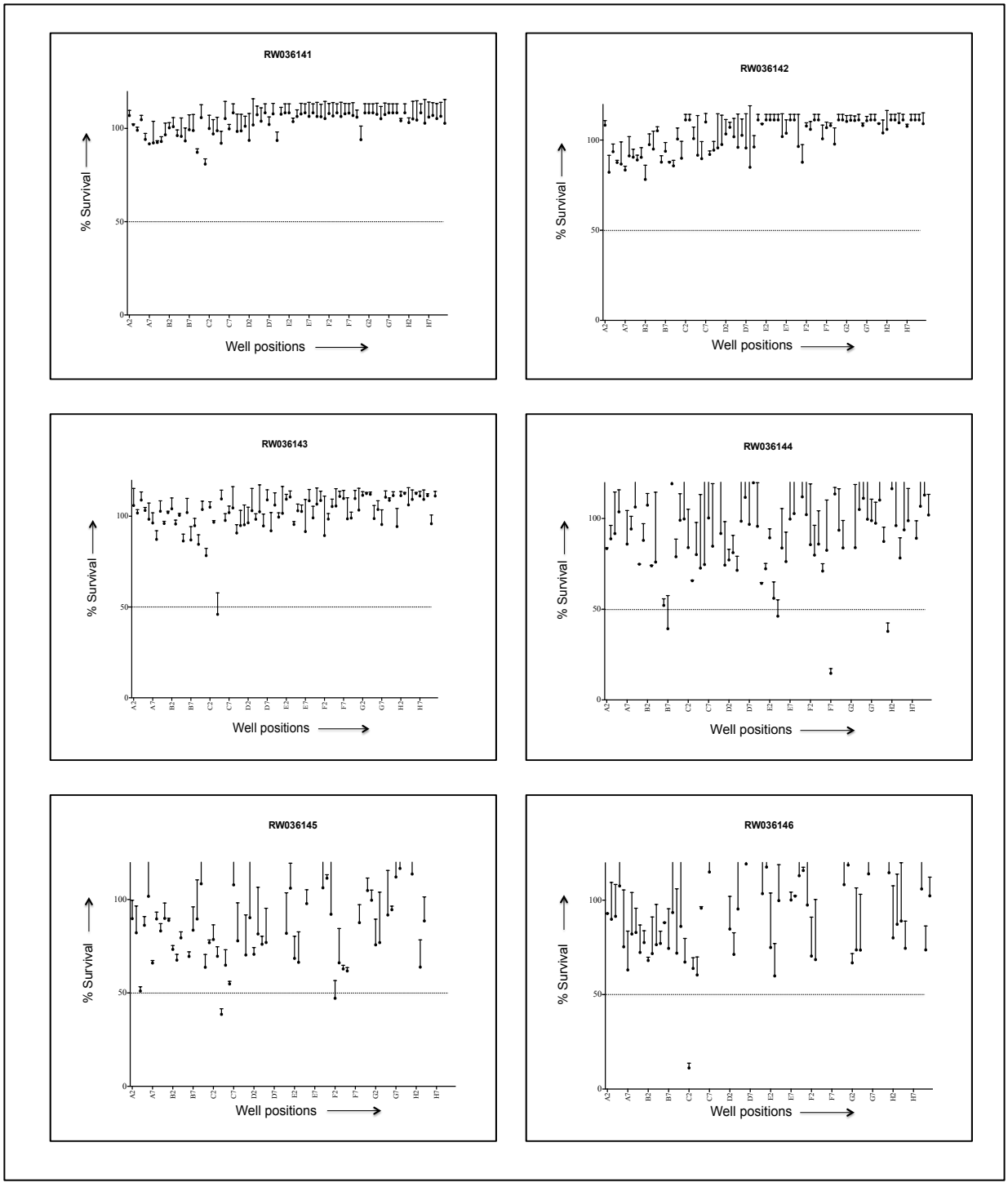


Figure 43: Graphs representing survival percentages of *M. smegmatis* (pSMT3_egfp) in the presence of various compounds from the compound library. Each graph is representative of a different compound plate as labeled from RW036141- RW036146. The X-axis of the graph represent the well numbers on the assay plate containing specific compounds, Y- axis has the survival percentages. The cut-off line for screening was taken at 50% survival rate in the presence of the compound. Z' factor- 0.5-0.7

All the "Hits" from the above-mentioned assay were considered as novel compounds for further study as none of them have been previously studied in context with *Mycobacterium tuberculosis*. The "Hits" attained from the above shown assay are as follows:

Plate Number	Position in Plate	Compound Name	Percentage Survival (%)
RW036143	C04	2-(-4-chloro-3-methylphenoxy)-5-nitro-1, 3-thiazole	37.62
RW036144	B07	N-[4-({[(3-ethoxybenzoyl)amino]carbothioyl}amino) phenyl]acetamide	26.3
	E04	1-{2-[(2,4-dicholorophenyl)(phenyl)methoxy]ethyl} 2-methyl-4,5-hydro-1H-imidazole	39.75
	F07	2-mesityl-1-oxo-1H-inden-3-yl-acetate	37.62
	G11	3-(2-fluorophenyl)-1-[4-(methylsufanyl) phenyl]-2-	25.81

		propen-1-one	
RW036145	C04	N- (4-butylphenyl)-N'- (2,2-dimethoxyethyl) thiourea	36.4
	F02	6-[5-2-pyridinyl)-4,5-dihydro-1H-pyrazol-3-yl]-2H-1,4-benzoxazin-3(4H)-one	40.45
RW036146	C02	2-{[4-amino-5-(2-cholorophenyl)-4H-1,2,4-triazol-3-yl] sulfanyl}-N- (2-ethyl-6-methylphenyl) acetamide	12.99

Table 3: The table mentioned above has all the "hits" from the compound screen, the highlighted ones have shown some relative study or chemical properties which could lead to possible understanding of the mechanism of action

1. RW036143

The plate had one hit with a 37.62% survival percentage. The compound identified was in the well C04. The chemical name of the compound is 2-(-4-chloro-3-methylphenoxy)-5-nitro-1, 3-thiazole, with the chemical structure as mentioned in Fig 44.

2. RW036144

The plate had four hits with two of them at a lower survival percentage of 26.3% and 25.81% for B07 and G11 respectively, while the other two were at 37.62% and 39.75% survival percentages for F07 and E04 respectively. The compound names are N-[4-({[(3-ethoxybenzoyl)amino]carbothioyl}amino)phenyl]acetamide (B07), 3-

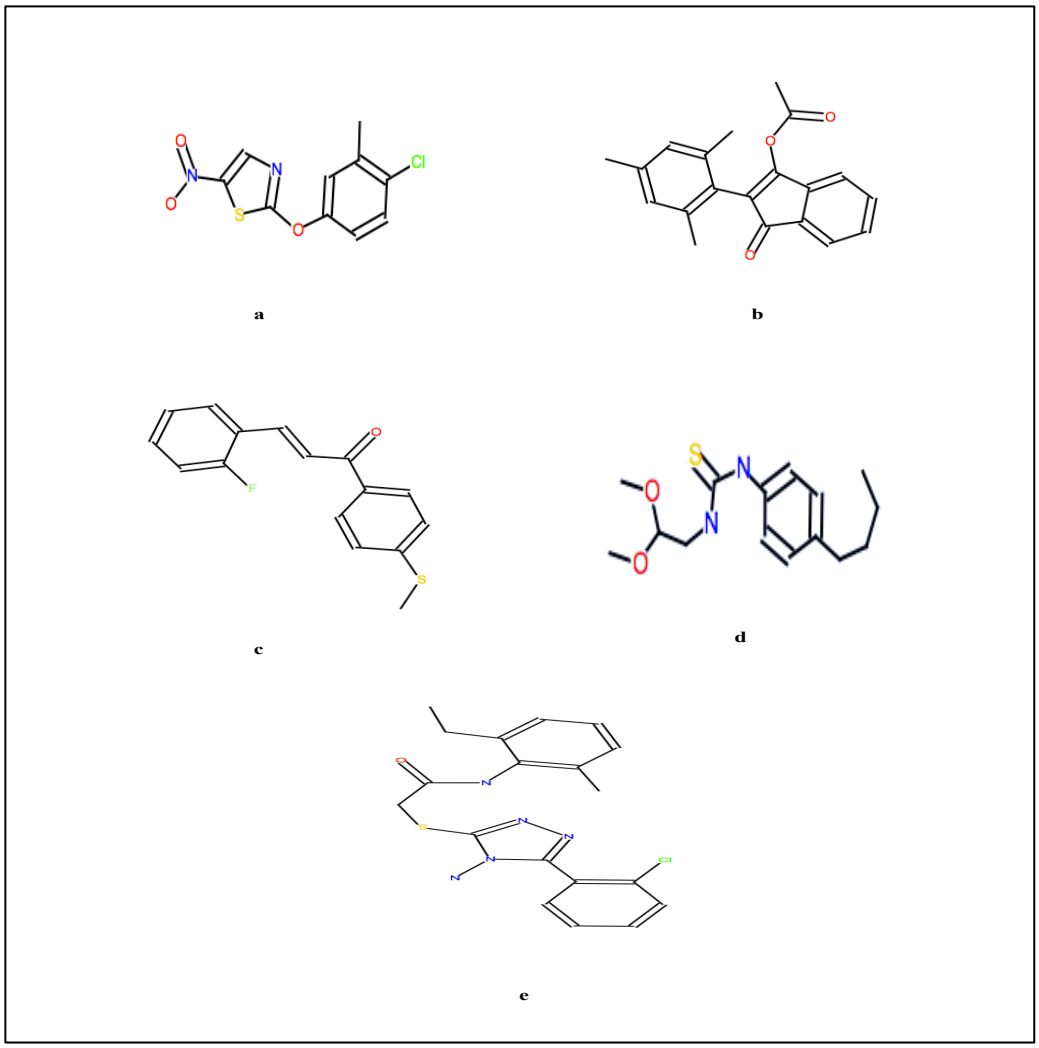
(2-fluorophenyl)-1-[4-(methylsufanyl) phenyl]-2-propen-1-one (G11), 2-mesityl-1-oxo-1H-inden-3-yl-acetate (F07) and 1-{2-[(2,4-dicholorophenyl)(phenyl)methoxy]ethyl}2-methyl-4,5-hydro-1H-imidazole (E04), with chemical structures as mentioned in Fig 44.

3. RW036145

The plate had two hits with a 36.40 % and 40.45% survival percentage in well C04 and F02. The chemical name of the compound is N- (4-butylphenyl)-N'- (2,2-dimethoxyethyl) thiourea and 6-[5-2-pyridinyl)-4,5- dihydro-1H-pyrazol-3-yl]-2H-1,4-benzoxazin-3(4H)-one, with the chemical structure as mentioned in Fig 44.

4. RW036146

The plate had one hit with a 12.99 % survival percentage. The compound identified was in the well C02. The chemical name of the compound is 2-{[4-amino-5- (2-cholorophenyl)-4H-1, 2,4-triazol-3-yl] sulfanyl}-N- (2-ethyl-6-methylphenyl) acetamide, with the chemical structure as mentioned in Fig 44.


a
b
c
d
e

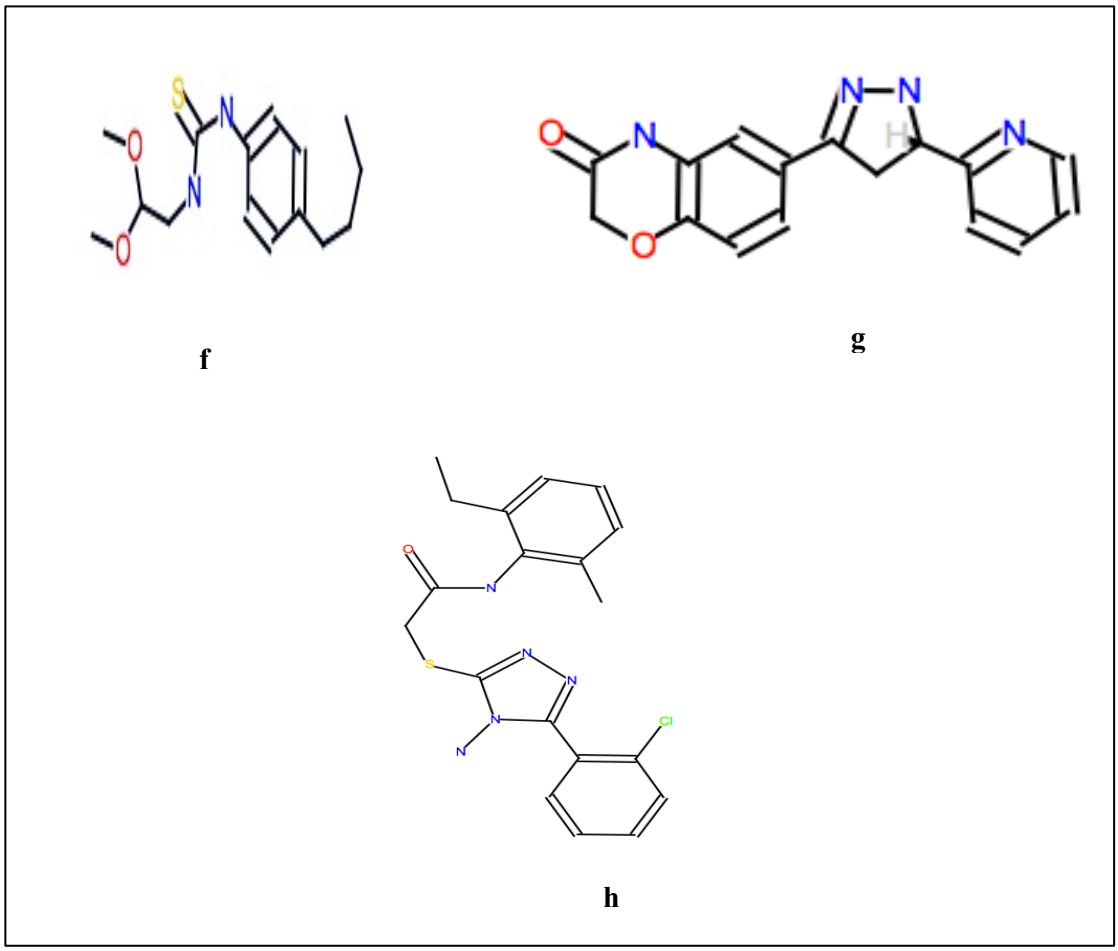


Figure 44: Compound hits

a.2-(-4-chloro-3-methylphenoxy)-5-nitro-1,3-thiazole ;

b.2-mesityl-1-oxo-1H-inden-3-yl-acetate;

c.3-(2-fluorophenyl)-1-[4-(methylsufanyl)phenyl]-2-propen-1-one ,

d.N-[4-({[(3-ethoxybenzoyl)amino]carbothioyl}amino)phenyl]acetamide

e.1-{2-[(2,4-dicholorophenyl)(phenyl)methoxy]ethyl}2-methyl-4,5-hydro-1H-imidazole

f.N-(4-butylphenyl)-N'-(2,2-dimethoxyethyl)thiourea,

g.6-[5-2-pyridinyl)-4,5- dihydro-1H-pyrazol-3-yl]-2H-1,4-benzoxazin-3(4H)-one

h.2-{[4-amino-5-(2-cholorophenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-ethyl-6-methylphenyl)acetamide.

5.4 HTS screen against FDA library

The FDA drug screen resulted in many “hits”, that is those drugs, which caused the survival percentage of *M. smegmatis* (pSMT3_egfp) to be at and below 50% value presented in Fig. 45. The “Hits” attained from the above shown assay had many generic antitubercular drugs along with different FDA drugs available for treating other diseases.

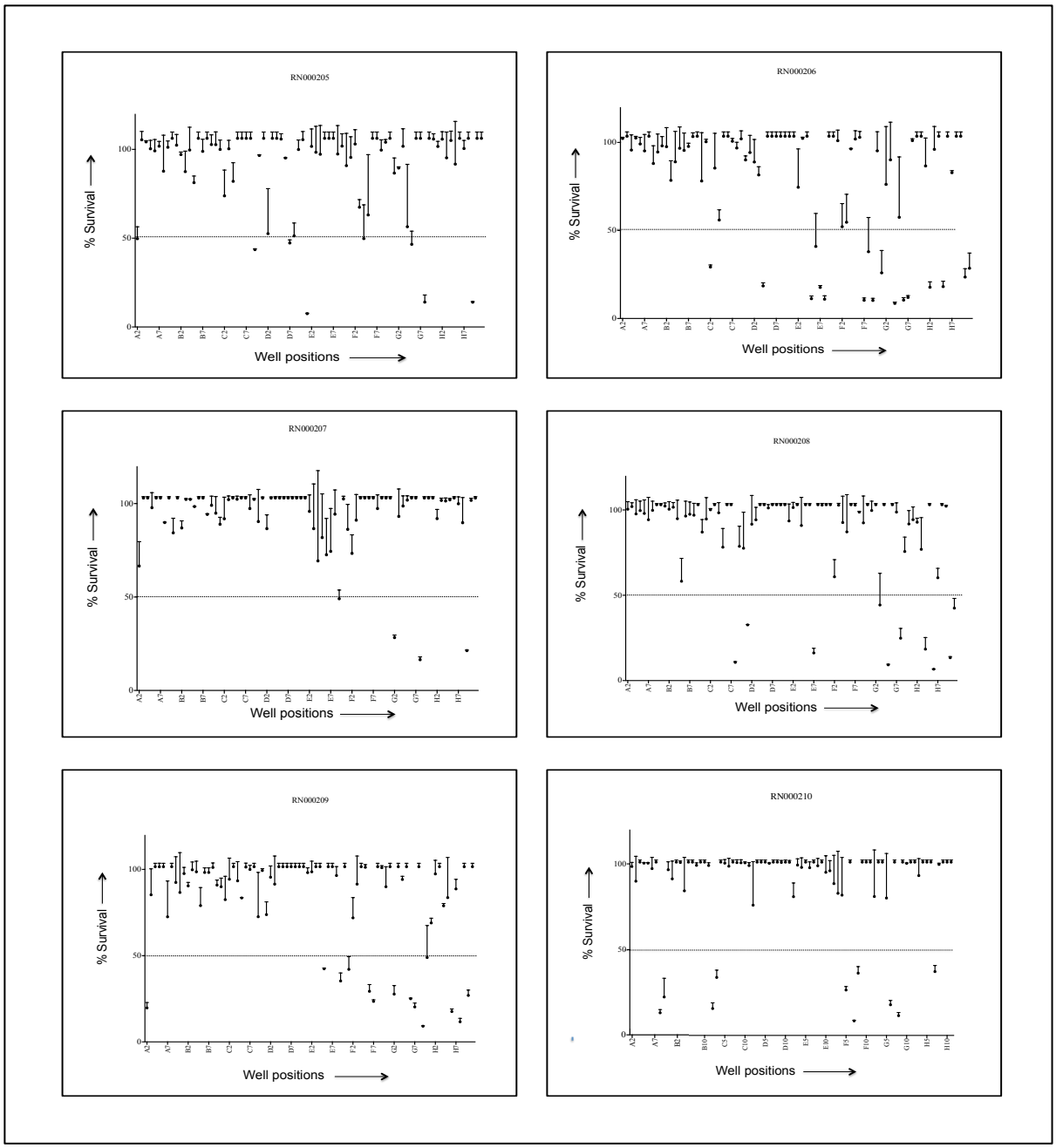


Figure 45: Graphs representing survival percentages of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library. Each graph is representative of a different compound plate as labeled from RN000205- RN000210. The X-axis of the graph represent the well numbers on the assay plate containing specific FDA drugs, Y- axis has the survival percentages. The cut-off line for screening was taken as 50% survival rate in the presence of the compound. Z' factor – 0.5-0.7

The "hits" mentioned below are all those drugs which produced a survival percentage of less than 50%. Out of the below mentioned drugs, several drugs were shortlisted on basis of novelty of use against *Mycobacterium* after thorough literature search and review (Table 4).

Plate Number	Position in plate	Drug Name	Percentage Survival (%)
RN000205	A2	Azaguanine-8	49.62614664
	A3	Allantoin	105.3027486
	C9	Ethambutol	43.52914306
	D2	Chloramphenicol	52.16180109
	D7	Troleandomycin	47.23993656
	D8	Pyrimethamine	51.44009912
	D11	Niclosamide	7.520399947
	G8	Miconazole	13.95385752
	H9	Thioridazine hydrochloride	13.96079893
RN000206	C2	R (-) Apomorphine hydrochloride hemihydrate	29.2392455
	D4	Ciprofloxacin hydrochloride monohydrate	18.45764194
	E5	Tioconazole	11.23998443
	E7	Mefloquine hydrochloride	17.6479618
	E8	Isoconazole	10.92490978
	F7	Astemizole	10.33337356
	G6	Chlorotetracycline hydrochloride	10.44025611

	G7	Tamoxifen citrate	11.96387987
	H2	Erythromycine	17.69592958
	H5	Josamycin	18.05917104
	H10	Dihydrostreptomycin sulfate	23.47853454
	H11	Gentamicine sulfate	28.46001794
RN000207	E9	Trimethoprim	49.14275185
	G2	Norfloxacin	28.31717438
	G8	Clemizole hydrochloride	16.49153847
	H9	Lomefloxacin hydrochloride	21.33615203
RN000208	C8	Clotrimazole	10.70325838
	C11	Fendiline hydrochloride	27.41299691
	E7	Perhexiline maleate	18.10390467
	G5	Econazole nitrate	9.02389887
	G8	Oxytetracycline dihydrate	25.53351592
	H4	Trifluoperazine dihydrochloride	17.59590399
	H6	Minocycline hydrochloride	7.293698781
	H10	Clofilium tosylate	15.58719476
RN000209	A2	Streptomycin sulfate	19.67469832
	E5	Metergoline	42.36107314
	E9	Bepridil hydrochloride	35.25149373
	E11	Benzbromarone	42.13418206
	F6	Methiothepin maleate	29.3687273
	F7	Clofazimine	23.52110751
	G2	Lidoflazine	27.76877416

	G6	Mitoxantrone dihydrochloride	25.00702933
	G7	GBR 12909 dihydrochloride	20.28253993
	G9	Dequalinium dichloride	9.077205452
	H6	Amikacin hydrate	17.57039091
	H8	Clomiphene citrate	11.67063694
	H10	Prochlorperazine dimaleate	26.97953685
RN000210	A9	Butoconazole nitrate	12.48838345
	A10	Amiodarone hydrochloride	24.80763778
	C2	Suloctidil	14.38444091
	C3	Zotepine	56.03883472
	F5	Linezolid	23.5113094
	F7	Meclocycline sulfosalicylate	8.915604769
	F8	Meclozine dihydrochloride	30.91774694
	G6	Tosufloxacin hydrochloride	19.33072913
	G8	Rifapentine	13.18458897
	H7	Primaquine diphosphate	35.23359113

Table 4: The table mentioned above has all the “hits” from the FDA drug screen, the highlighted drugs are the ones, which were researched and found to be novel as per the literature. Therefore the drugs were taken further to do a dose response analysis.

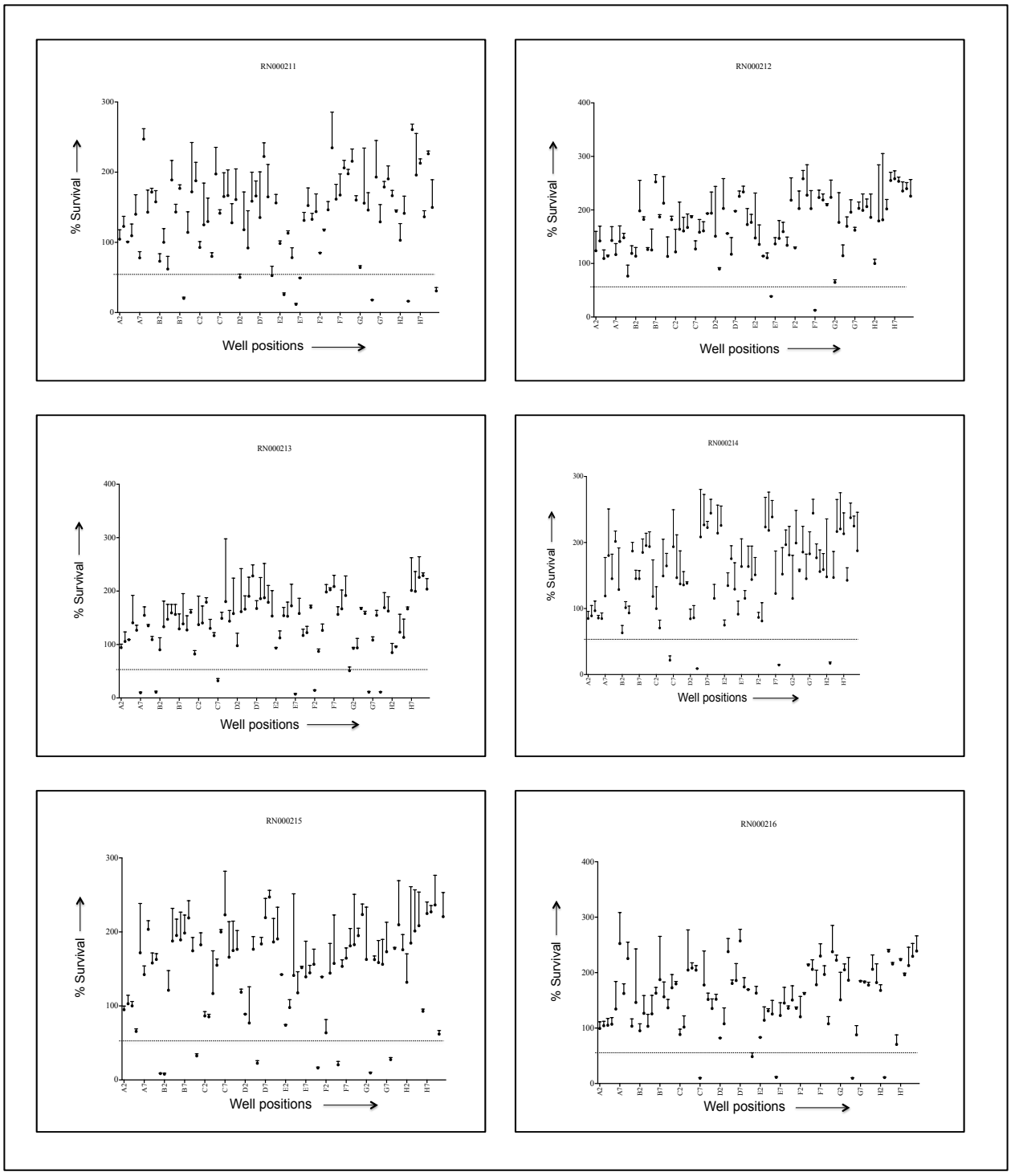


Figure 46: Graphs representing survival percentages of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library. Each graph is representative of a different compound plate as labeled from RN000211- RN000216. The X-axis of the graph represent the well numbers on the assay plate containing specific FDA drugs, Y- axis has the

survival percentages. The cut-off line for screening was taken at 50% survival rate in the presence of the compound. Z' factor – 0.5-0.8

The FDA drug screen resulted in many "Hits", which were considered to be all those drugs, which caused the survival percentage of M. smegmatis (pSMT3_egfp) to be at and below 50% value. The "Hits" attained from the above shown assay had many generic antitubercular drugs along with different FDA drugs available for treating other diseases. The "hits" mentioned below are all those drugs which produced a survival percentage of less than 50%. Out of the below mentioned drugs, several drugs were shortlisted on basis of novelty of use against Mycobacterium after thorough literature search and review. The Highlighted drugs in the below mentioned table would be taken for further analysis for dose response analysis.

Plate Number	Position in plate	Drug Name	Percentage Survival (%)
RN000211	B8	Vancomycin hydrochloride	20.21
	D2	Fluoxetine hydrochloride	50.13
	D10	Imipenem	52.635
	E6	Rifampicin	11.645
	E7	Ethionamide	49.29
	G5	Tobramycin	17.85
	H4	Pentamidine isethionate	16.19
	H11	Prenylamine lactate	30.765
RN000212	E6	Carbadox	38.425
	F7	Demecarium bromide	12.615
RN000213	A7	Oxiconazole Nitrate	9.565

	A11	Pinaverium bromide	10.705
	C7	Nisoldipine	32.1
	E7	Thimerosal	7.1
	F11	Cefmetazole sodium salt	51.06
	G6	Methyl benzethonium chloride	10.815
	G9	Benzethonium chloride	10.815
RN000214	D4	Demeclocycline hydrochloride	9.045
	F8	Alexidine dihydrochloride	14.485
	H3	Propidium iodide	17.14
RN000215	A11	Sulconazole nitrate	8.695
	B2	Auranofin	7.865
	B10	Moxalactam disodium salt	32.585
	D5	Novobiocin sodium salt	22.62
	E10	Clinafloxacin	16.195
	G3	Raloxifene hydrochloride	9.535
	G8	Paromomycin sulfate	27.56
RN000216	C7	Fluspirilen	9.58
	E6	Thonzonium bromide	11.115
	G5	Phenoxybenzamine hydrochloride	9.44
	H3	Toremifene	10.875

Table 5: The table mentioned above has all the “hits” from the FDA drug screen, the highlighted drugs are the ones, which were researched and found to be novel as per the literature.

5.5 Dose response against selected drugs

The screening assay conducted before presented all of the selected drugs to be active at the concentration of 0.5mM. The dose response assay was performed to check for lower concentrations at which inhibition could occur.

As shown in fig. 47, Meclocycline sulfosalicylate showed activity at a much lower concentration of 0.00205mM. While drugs such as Clomiphene citrate and sulocitidil showed a dip in the growth of the bacillus at a concentration of 0.0555mM. Tamoxifen citrate showed an inhibitory concentration of 0.166mM. While for Fendiline hydrochloride the concentration range seems not to have covered the inhibitory concentration that leads us to believe that the primary concentration of drug used that is 0.5mM could be the minimum inhibitory concentration for it.

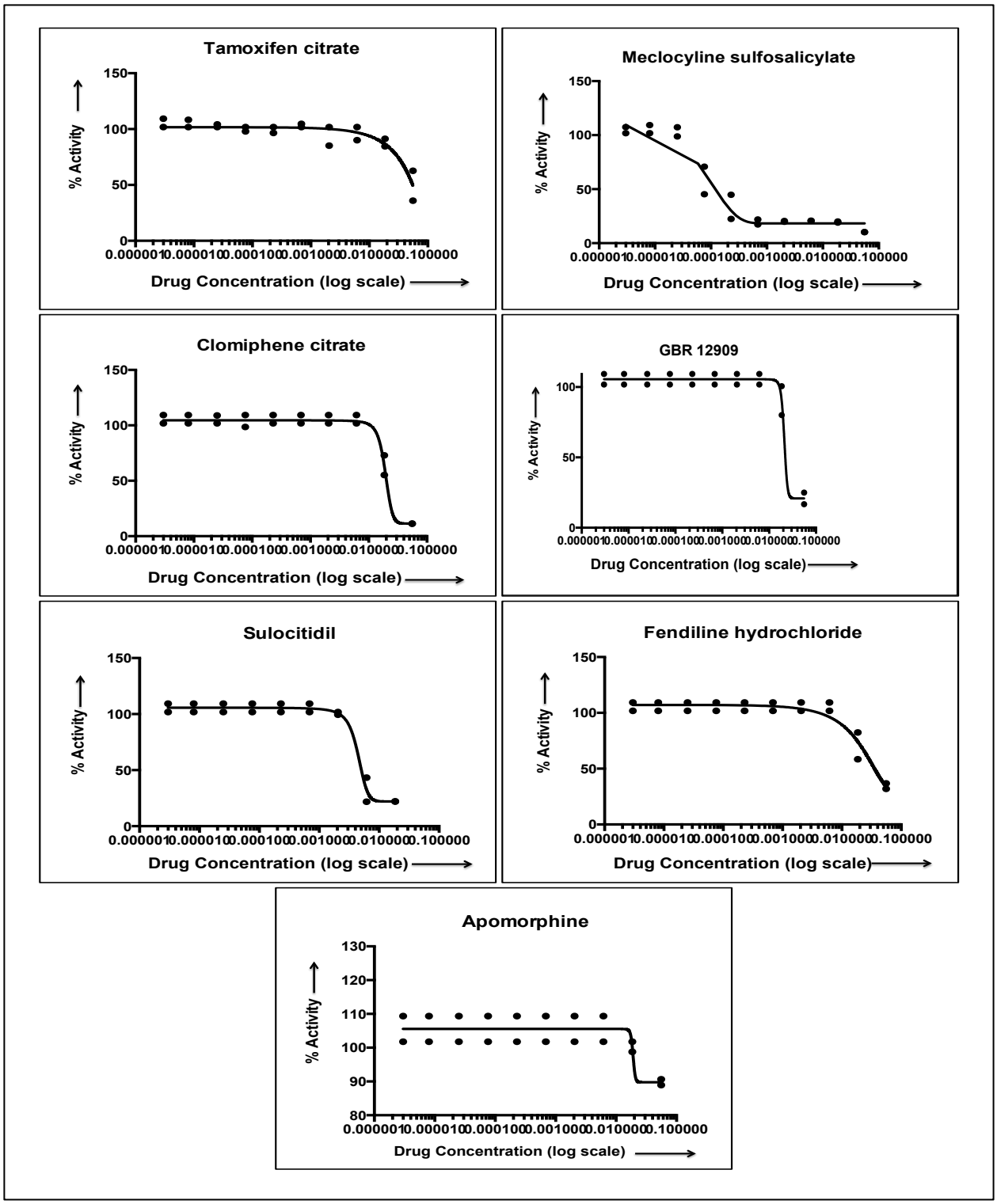


Figure 47 : Graphs representing the dose response curves of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library, which showed activity in the first round of screening. Z' factor- 0.6-0.8

6. DISCUSSION

6.1 Diversity Library

6.1.1 2-(-4-chloro-3-methylphenoxy)-5-nitro-1,3-thiazole

Compounds similar to the said thiazole with different side groups have shown antibacterial activity. Nitrothizolyl is an antiparastic, which has been seen to be mircobicidal for *M. tuberculosis*, acting on it by disrupting its membrane potential. Analogues of 5- nitrothioaolyl and its derivatives are very potent against *M. tuberculosis*. Therefore the said compound in relation might have a similar mode of action due to its structure similarity and potency against *Mycobacterium* sps. (Jeankumar *et. al.*,2012)

6.1.2 2-mesityl-1-oxo-1H-inden-3-yl-acetate

The compound is inhibits the complement system and biosynthesis of prostaglandins (Asghar *et. al.*,1986, Van der Goot *et. al.*,1984). Interestingly it is known to act as an insecticide against pear psylla, a sap-feeding insect (family *cicada)* due to its surfactant activity, which is inhibitory for the insect (Formigoni, 1980). In humans there are four surfactant proteins (SP-A to D). Alveolar surfacatants interact with Mycobacterial surface proteins via their CRD domains modulating the bacterial viability, transcriptional progress and uptake and fate of the microbe in macrophages.

The surfactants initiate ingestion of infected epithelial cells and free bacilli by the macrophage. The disruption of surfactant metabolism causes the progression of tuberculosis from latent to a secondary infection (Chroneos *et. al.*,2009). The protein SP-D works by binding and masking the terminal mannose cap of ManLAM on the bacterial surface directing phagocytosis and promoting phagosome maturation. (Ferguson *et. al.*, 2006). The modulatory activity of this surfactant like compound could be an important step in understanding its mode of action.

6.1.3 1-{2-[(2,4-dicholorophenyl)(phenyl)methoxy]ethyl}2-methyl-4,5-hydro-1H-imidazole

Imidazolines have been known to act as antagonists to muscarinic M3 receptors, which are mainly acetylcholine receptors. These up regulate the phospholipase C and regulate intracellular calcium levels for signaling pathways. No direct link has been proved as yet regarding the regulation of calcium levels with infectivity with *Mycobacterium* but it could be one of the mechanisms through which the compound could be acting (Unno *et. al.*,2005)

6.1.4 3-(2-fluorophenyl)-1-[4-(methylsufanyl)phenyl]-2-propen-1-one

The chalcones, are an important category of antimycobacterials; 23 synthesised chalcones (including 4-methyl thiochalcones) have shown a 98% reduction in relative light unit (RLU) in *M. tuberculosis* (Sivakuma *et. al.*,2010). They are mainly effective against phosphotyrosine phosphatase (PTP), which are majorly involved in cell

signaling pathways. In *Mycobacterium* there are two PTPs, PtpA and PtpB. PtpA uses VPS3313 as a substrate to avoid fusion of the phagolysosome therefore making it and is an essential protein for persistence of the bacillus. Attenuated growth of Mycobacterium has been seen on inactivation of the PtpA gene (Mascarello *et. al.*, 2010; Chiaradia *et. al.*, 2008). The nature and position of the substituent in the ring A and the hydrophobicity and planarity of napthyl groups ring B are seen as predominant factors for the inhibition of Ptp A (Chiaradia *et. al.*, 2008). Chalcones are competitive inhibitors of PtpA and B, where polar interactions play a key role in orienting the molecule within the protein-binding site. The compound tends to bind in a narrow channel close to the catalytic site at a cysteine residue at the 160 positions. (Chiaradia *et. al.*,2012).

6.1.5 N-(4-butylphenyl)-N'-(2,2-dimethoxyethyl)thiourea

The compound has yet not been worked upon directly. But related compounds such as N-(4-butylphenyl)-N'-[1-(methoxymethyl)propyl] thiourea , is known to have inhibitory activity against glycogen synthase kinase 3 (GSK-3) by inhibiting its transport from the cell nucleus to cytoplasm. GSK-3 has been involved in causing major diseases such as alzheimers, cancer etc. (Yook *et. al.*,2010). In *Mycobacterium*, glucosyltansferase uses UDP- glucose as a sugar donor to elongate the α (1 to 4) glucans. It is mainly implicated in the biosynthesis of 6-O-methylglucosyl lipopolysaccahrides. The gene coding for glycogen synthase in *Mycobacterium*, Rv3032 does not cause lethality in the organism if disrupted but does affect the multiplication and persistence and increases its sensitivity to validamycin A; which is a TreS inhibitor. It has a supporting gene *glgA*, which manages to help the bacillus

survive, but knocking both these genes causes organism death leading us to the fact that atleast one functional copy of either gene is necessary for its survival.

Another compound similar to the said compound is an inhibitor of epoxy hydrolase and is used for the treatment of hypertension (Kroetz *et. al*,2003). *Mycobacterium* has six epoxide hydrolases, which has a role in converting epoxides into trans dihydrodiols (Biswal *et. al.*,2008). The enzymes play a role in virulence, detoxification and regulation of signaling molecules and defense systems in mammals. (Johansson *et. al.*,2005)

The exact mechanism of action has not been reported, but it could be possible that since similar compounds have the said inhibitory actions they could be similar in mode of action for *Mycobacterium* as well.

6.1.6 2-{[4-amino-5-(2-cholorophenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-ethyl-6-methylphenyl)acetamide

Acetamides have been known to have an antitubercular effect the mode of action of the compound could be predicted. Acetamides act against the cell wall, disrupting the cell wall biosynthesis process. Sulfonyl acetamide derivatives are a new class of antibiotics that mimic the transition state of β-ketoacyl synthase (KAS) of fatty acid biosynthesis (Ghosh *et. al.*,2008). N-octanesulfonylacetamide (OSA) causes reduction in the accumulation of mycolic acids in BCG and *Mycobacterium Avium* complex (MAC) by affecting the FAS system. Although OSA also acts on the cell wall similar to isoniazid it has been seen to inhibit isoniazid resistant *M. tuberculosis* and MAC (Parish et. al.,2001). N- phenylphenoxyacetamide derivatives inhibits a transcriptional

repressor, *EthR* which causes an innate resistance to second line drugs mainly ethionamide (Flipo et. al.,2012). Ethionamide like isoniazid also acts on the cell wall but via a different mechanism, that is by getting activated through an FAD containing enzyme which catalyses NADPH and converts ETA to ETA-S-oxide through an oxygen dependant monoxygenation. (Parish *et. al.*,2001). Although no exact structure matches were obtained against the compound alongwith the absence of any specific biological roles, the compound appears to be novel.

6.2 FDA Drugs

The FDA drugs which came out as successful inhibitors of *M. smegmatis* were a varying mixture ranging from anti cancer drugs to drugs used to treat skin conditions. Tamoxifen citrate is a lipophilic drug belonging to a class of non-steroidal triphenylethylene derivatives (Elnaggar *et. al.*,2009) and is a hormone therapy drug that is currently being prescribed for breast cancer chemotherapy causing a decrease in the proliferation of breast tissue. 45% of patients treated with tamoxifen showed a decrease in new tumor incidence after 5 or more years of use while 28% had decrease in benign tumors and lesions (De Souza Sales Jr *et. al.*,2007). It also has been shown to decrease risk of heart disease and osteoporosis, by decreasing the level of TNF R2, which plays a role in TNF α induced cardiac injury. Although the major mechanism of action in protecting the cardiac tissue is by increasing mitochondrial respiration, which is the major source of energy for the cardiac tissue. It does so by increasing the superoxide scavenging activity in mitochondria (Zhao *et. al.*,2006). Clomiphene citrate, which belongs to the same class as tamoxifen citrate is used to induce ovulation. Although a study comparing the efficacy of both these drugs declared

tamoxifen to be more effective as a hormone therapy drug to induce ovulation leading to more number of pregnancies and lower rates of miscarriages (*Boostanfar et. al.*,2001).Clomiphene citrate is although a safer choice to use as a fertility drug and acts as an estrogen agonist as well as antagonist. Agonistic properties of the drug occur when the endogenous estrogen level is very low while in other cases it acts as competitive estrogen antagonist. Clomiphene binds to nuclear estrogen receptors for a longer times interfering with the regular cycling and replenishment of the ER; while at the hypothalamic level it decreases the hypothalamic estrogen receptors causing an overcompensatory response which increases the gonadotropin releasing hormone (GnRH) causing an increase in ovarian follicular activity (Pfeifer *et. al.*,2013). It has also been traditionally used in the treatment of non-obstructive oligospermia and reversal of hypogonadism via gonadotropin secretion (*Ioannidou-Kadis et. al.*,2006; Kaminetsky and Hemani,2009). Another similar drug Raloxifen which is also a competitive estrogen receptor modulator (*Kathryn Senior*, 1999) was compared with Tamoxifen which again showed better results for estrogen replacement therapy by decreasing the circulating estrogen and decreasing chances of osteoporotic fractures in menopausal women (Khovidhunkit and Shoback,1999); but at a higher risk of causing endometrial cancer on long term use (Boss *et. al.*, 1997). The drug raloxifen has been seen to act as an antiviral agent against patients with chronic hepatitis C by directly inhibiting the replication system of the virus (Furusyo *et. al.*,2012).

Several antibacterial and antiseptics also were active against *Mycobacterium* such as meclocylcline sulfosalicylate which is majorly used as a topical ointment for treating skin conditions such as acne (Borglund *et. al.*,1991) and mostly used in combination with other drugs such as benzoyl peroxide to observe 82% success rate and decrease in skin lesions (Knutson *et. al.*,1981). Demeclocycline hydrochloride is a tetracycline

derivative highly effective against microbes with a very good bioavailability (Dong *et. al.*,2013). Although being an Anti Diuretic Hormone (ADH) antagonist it is majorly used to increase excretion of solute free water to treat patients with inappropriate hyper secretion of ADH (Garwicz,1976) and cancer patients who develop hyponatremia during treatment (Trump,1981). Benzethonium chloride is another drug, which is used as a preservative for vaccines (Olson *et. al.*,1964) and a disinfectant in hospitals. It has also been seen that it acts as an inhibitor of HERG potassium channels, which could cause risks of cardiac arrythmia (Long *et. al.*,2013). In recent times through similar protocol of HTS the drug has shown a novel anticancerous activity against human cancer cell lines inducing apoptosis and activation of caspases. It caused loss of membrane potential on the mitochondrial membrane and and increase in calcium (Ca^{2+}) level leading to cell death (Yip *et. al.*,2013).

GBR 12909 dihydrochloride commercially known as Vanoxerine is an antiarrythmatic drug used to terminate induced atrial flutter and atrial firbrillation (AF). Earlier use involved treatment for Parkinson's and depression, but had a low efficacy while in case of reducing atrial flutter it has an excellent efficacy and safety profile as it has multichannel blocking properties (Cakulev *et. al.*,2012). It interrupts an atrial flutter by blocking the circulating reentrant wave front in an area of slow conduction (Szasz *et. al.*,2007). It has also been seen to be useful in treating cocaine addiction by attaching to the dopamine transporter (DAT) for a longer time compared to cocaine (Rothman *et. al.*,2008). Cocaine has a reinforcing effect mediated by dopamine on dopaminergic neurons. It binds to monoamine transporters inhibiting reuptake of their respective neurotransmitter (that is dopamine) therefore increasing the extracellular level of dopamine causing stimulation of postsynaptic dopamine

receptors (Andersen, 1989). R(-) Apomorphine hydrochloride hemihydrate is a D1 and D2 dopaminergic agonist, useful for diagnosing idiopathic Parkinson's disease and other Parkinson plus syndrome (Warraich *et. al.*,2009). It acts as an indicator of dopaminergic responsiveness and is a dopamine receptor agonist with potent anti-Parkinson action (Hughes *et. al.*,1990). Apomorphine is known to alleviate akinesia (slowness of initiation), rigidity and tremor (Muguet *et. al.*,1995) when administered subcutaneously(Frankel *et. al.*,1990).

Sulocitidil among many other drugs such as aspirin, dipyridamole, sulfinpyrazone etc. is a platelet-inhibiting drug. It is mainly used to elevate thrombosis, which is a cause for strokes sometimes followed by transient ischemic attacks (neurological dysfunction due to loss of blood flow) (Gent *et. al.*,1985). It is a potent antiaggregating drug whose common mechanism of action is inhibition of phosphodiesterase activity and increase of cyclic AMP (Roba *et. al.*,1975). It has been understood that sulocitidil is also a calcium antagonist, since calcium ions play a major role in PG_2 synthesis it causes a slow efflux of calcium into endothelial cell leading to prolonged stimulation and release of prostacyclin (PG_1); a potent inhibitor of platelet aggregation (Boeynaems *et. al.*,1987). Fendiline hydrochloride is another calcium antagonist (Nayler and Horowitz, 1983) used as an anti-anginal agent for the treatment of coronary heart disease. It inhibits trans membrane calcium current and causes smooth muscle relaxation therefore providing cardio protection (Bayer and Manhold,1987). It has been studied to have possible anti cancer properties as it inhibits downstream signaling of constitutively active K-Ras, which is important for cell proliferation, cell growth, differentiation and survival. In oncogenic cells mutant Ras protein is produced, which when blocked has been understood to block cancer cell proliferation in cancer cell lines expressing the mutant Ras protein (Van der

Hoeven *et. al.*,2013). Pinaverium bromide is a quaternary ammonium compound used to treat intestinal motor disorder and irritable bowel syndrome. It decreases colonic response to food and has a high efficiency to postprandial (after eating) inhibition of muscular contractions (Bouchouchal *et. al.*,2000; Christen, 1990). It is a L- type calcium blocker which binds to α-1 subunit of Ca^{2+} channel and inhibits the contraction of smooth muscles induced by cholecystokinin (CCK) (Boyer *et. al.*,2001).Another calcium antagonist is nisoldipine which is helpful in treating severe ventricular dysfunction. It has systemic coronodilatory function helpful to patients with left ventricular dysfunction (Rousseau *et. al.*,1994). Its administration has lead to improved exercise tolerance, improved diastolic filling probably related to improvement of chronic ischemic areas (Pouleur And Rousseau, 1993).

Interestingly, drugs that have shown activity against *M. smegmatis* have repetitive classes of drugs having similar modes of action. This could be an indicative of specific drug target areas influencing cell death in *Mycobacterium*. The mechanism of action of individual drug against *Mycobacterium* has yet to be explored, but it is interesting to see that the drugs are grouped in terms of mode of action and the target on the host cell. The only possible known mechanism of action studied till date is that of calcium channel blockers which are accounted for among the drug "hits" reported. Calcium ions and Mycobacterium have had a long-standing relationship involving its role in pathogenesis and host-pathogen interaction. Calcium plays an important role in the fusion of phagosome with the lysosome in neutrophils and macrophages (Vergne *et. al.*,2003). Calcium ions along with potassium ions have a significant role in phagolysosome biogenesis and killing of the bacillus. Both ions depend on active transport processes for transport across membranes. When the bacillus is phagocytosed the phagosome is made up of the plasma membrane that is rich in

calcium and potassium ion transport pumps. The bacteria use these pumps to transport the calcium and potassium ion pumps to pump out the ions from within the phagosome to the cytoplasm of the cell. During the fusion of the phagosome to the lysosome the low concentration of calcium and potassium ions prevents acidification of the phagolysosome due to absence of hydrogen ion influx across a concentration gradient; therefore protecting the bacillus form getting killed (Martins *et. al.*,2008). it has been seen that blockage of L- and R- type voltage gated calcium channel (VGCC) plays a regulatory role for immune response by activation of calcium channel dependent mechanism favoring inflammatory response. In vivo blockage of VGCC in *M. tuberculosis* infected mice using antibodies increase the intracellular calcium and decreased bacterial loads (Gupta *et. al.*,2009). The exact mechanisms for the drugs and compound "hits" are still to be explored through various strategies, but the assay using a fluorescent strain of *M. smegmatis* has been designed and optimized successfully to give a robustly and reliably screen out compounds against *Mycobacterium*.

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Figure 35. The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin

Figure 36. The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds and DMSO controls alogwith media controls. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

Figure 37. The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds and DMSO controls (1.25%, 2.5%, 5%) alongwith media controls. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

Figure 38. The above assay plates depict the setup for *M. smegmatis* (mc^2155) and *M. smegmatis* (pSMT3_egfp) with serially diluted standard antibiotics by 2 folds. All conditions were performed in duplicate rows to maintain assay validation. INZ – Isoniazid, PYZ – Pyrazinamide, EMB – Ethambutol, RIF – Rifampin.

Figure 39-The above assay plates depicts the setup for screening library compunds and drugs with the columns 1 and 12 as positive controls while the columns 2-11 had different compounds/drugs in each well.

Figure 40. The above assay plates depict the setup for a dose response study of selected FDA approved drugs (A-G) serially diluted (3-fold) from 2-11; with column 1 as a positive control and column 12 as a negative control. A. Tamoxifen citrate, B. Meclocycline sulfosalicylate, C. Clomiphene citrate salt, D. GBR 12909 dihydrochloride, E. Sulocitidil, F. Fendiline hydrochloride, G. R- (-)- Apomorphine hydrochloride hemihydrate

Figure 41. Graphs representing survival percentages of *M. smegmatis* (mc^2155), pSMB_egfp and pMind_gfp. Survival curves for pMind_gfp are represented for varying concentration of tetracycline (2.5ng/ml – 25ng/mL) with an M. smegmatis (mc^2155) control. Survival curves for pSMT3_egfp represent varying conditions of hygromycin and Tween-80 with M. smegmatis (mc^2155) as a control.

Figure 42-Graphs representing survival and inhibition percentages of M. smegmatis (pSMT3_eGFP) against standard drugs like Isoniazid, Ethambutol, Pyrazinamide and Rifampicin over dilutions of 2 folds. a. and b. Representing the survival percentages during early optimization of cell concentration (1:1 and 2:1 respectively). c. and d. represent the drug standards run during the compound library screening. Z' factor – 0.6-0.7.

Figure 43.Graphs representing survival percentages of *M. smegmatis* (pSMT3_egfp) in the presence of various compounds from the compound library. Each graph is representative of a different compound plate as labeled from RW036141- RW036146. The X-axis of the graph represent the well numbers on the assay plate containing

specific compounds, Y- axis has the survival percentages. The cut-off line for screening was taken at 50% survival rate in the presence of the compound. Z' factor- 0.5-0.7.

Figure 44. Compound hits
a.2-(-4-chloro-3-methylphenoxy)-5-nitro-1,3-thiazole ;
b.2-mesityl-1-oxo-1H-inden-3-yl-acetate;
c.3-(2-fluorophenyl)-1-[4-(methylsufanyl)phenyl]-2-propen-1-one ,
d.N-[4-({[(3-ethoxybenzoyl)amino]carbothioyl}amino)phenyl]acetamide
e.1-{2-[(2,4-dicholorophenyl)(phenyl)methoxy]ethyl}2-methyl-4,5-hydro-1H-imidazole
f.N-(4-butylphenyl)-N'-(2,2-dimethoxyethyl)thiourea,
g.6-[5-2-pyridinyl)-4,5- dihydro-1H-pyrazol-3-yl]-2H-1,4-benzoxazin-3(4H)-one
h.2-{[4-amino-5-(2-cholorophenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-ethyl-6-methylphenyl)acetamide

Figure 45. Graphs representing survival percentages of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library. Each graph is representative of a different compound plate as labeled from RN000205- RN000210. The X-axis of the graph represent the well numbers on the assay plate containing specific FDA drugs, Y- axis has the survival percentages. The cut-off line for screening was taken as 50% survival rate in the presence of the compound. Z' factor – 0.5-0.7

Figure 46. Graphs representing survival percentages of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library. Each graph is representative of a different compound plate as labeled from RN000211- RN000216. The X-axis of the graph represent the well numbers on the assay plate containing specific FDA drugs, Y- axis has the survival percentages. The cut-off line for screening was taken at 50% survival rate in the presence of the compound. Z' factor – 0.5-0.8

Figure 47. Graphs representing the dose response curves of M. smegmatis (pSMT3_egfp) in the presence of various compounds from the FDA drug library, which showed activity in the first round of screening. Z' factor- 0.6-0.8

Table 2. Representing the antibiotics used for tuberculosis, with the MIC values, gene and gene products in *Mycobacterium* (Da Silva and Palomino, 2011)

Table 3. The table mentioned above has all the "hits" from the compound screen, the highlighted ones have shown some relative study or chemical properties which could lead to possible understanding of the mechanism of action.

Table 4. The table mentioned above has all the "hits" from the FDA drug screen, the highlighted drugs are the ones, which were researched and found to be novel as per the literature. Therefore the drugs were taken further to do a dose response analysis.

Table 5. The table mentioned above has all the "hits" from the FDA drug screen, the highlighted drugs are the ones, which were researched and found to be novel as per the literature.