CHEMICAL GENETIC APPROACHES TO COMBAT MULTIPLE DRUG RESISTANCE IN GRAM-NEGATIVE BACTERIA

Ph.D. THESIS

By

TAPAS BHATTACHARYYA

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)
DECEMBER, 2015

CHEMICAL GENETIC APPROACHES TO COMBAT MULTIPLE DRUG RESISTANCE IN GRAM-NEGATIVE BACTERIA

A THESIS

Submitted in partial fulfilment of the
Requirements for the award of the degree
of

DOCTOR OF PHILOSOPHY
in

BIOTECHNOLOGY

by

TAPAS BHATTACHARYYA

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)
DECEMBER, 2015

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"CHEMICAL GENETIC APPROACHES TO COMBAT MULTIPLE DRUG RESISTANCE IN GRAM-NEGATIVE BACTERIA"** is in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from December, 2009 to December, 2015 under the supervision of Dr. Ranjana Pathania, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(Tapas Bhattacharyya)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(Ranjana Pathania)
Supervisor

Date

ABSTRACT

Decline in the antibacterial drug discovery along with the emergence of multiple drug resistance in Gram negative bacterial pathogens poses a serious threat to the human health. Over a period of last 50 years, only a few antibacterial drugs have been introduced to the market and most of the clinically useful old antibiotics have become ineffective against many bacterial pathogens. Many of the infectious diseases, in particularly nosocomial (hospital acquired) infections caused by the Gram negative bacteria are almost incurable. *Acinetobacter baumannii*,the leading cause of hospital acquired infection worldwide is an example of such a grave situation. Various mutants of the organism are resistant to almost all the antibiotics available in the market. Discovery of novel antibacterial molecules with new therapeutic targets and reviving the efficacy of old antibiotics are the only solution of the problem. This study is an endeavor to that direction by employing a small molecule library. Phenotype based whole cell screening was adopted for screening a small molecule library of 10956 molecules against Gram negative bacteria. Screening led to the identification of IITR06144, a small molecule which is effective against a broad range of bacterial pathogens. The mode of action of the molecule was investigated by using molecular genetic techniques. The broad spectrum molecule was found to interfere with bacterial cell division. It restricts two daughter cells to separate from each other during cell division by inhibiting FtsZ, a major protein involved in septum formation during cytokinesis. In another approach, 8000 molecules were screened to discover a novel efflux pump inhibitor (EPI) of AbeM, a proton driven multidrug transporter in *A. baumannii*. Fluoroquinolones are the major substrates of AbeM and expression of this efflux pump confer fluoroquinolone resistance in *A.baumannii.* The objective of the study was to identify a molecule which can potentiate the efficiency of fluoroquinolones against the drug resistant *A.baumannii*. Elevated resistance of *E.coli* KAM32 (devoid of major efflux pumps) harboring multiple copies of *abeM* in a multicopy plasmid has been exploited for the screening. IITR08027, a small molecule (not inhibitory by its own) has been identified which causes a significant fold (16-32 fold) of reduction in Minimum Inhibitory Concentrations (MICs) of both ciprofloxacin and norfloxacin against clinical isolate of *A.baumannii* express AbeM or its homologues. The small molecule has been characterized and established as an efflux pump

inhibitor. The advantage of using the molecule in combination with ciprofloxacin against fluoroquinolone resistant *A.baumannii* was evaluated by chemical genetic studies.

LIST OF PATENTS AND PUBLICATIONS

PATENT

- Novel Efflux Pump Inhibitor (EPI) of multidrug transporter AbeM in *Acinetobacter baumannii*. (In the process of applying for Indian provisional patent)

PUBLICATION

- Antibacterial potential of a small peptide from Bacillus sp. RPT-0001 and its capping for green synthesis of silver nanoparticles. Patil SD, Sharma R, **Bhattacharyya T**, Kumar P, Gupta M, Chaddha BS, Navani NK, Pathania R. J Microbiol. 2015 Sep;53(9):643-52. doi: 10.1007/s12275-015-4686-3. Epub 2015 Aug 1.
- Screening of a small molecule library identifies a novel efflux pump inhibitor against AbeM, a multidrug efflux pump in Acinetobacter baumannii. **Bhattacharyya T**, Sharma A, Akhter J Pathania R. (Communicated to Journal of Antimicrobial Chemotherapy)

INTERNATINAL CONFERENCES

- A novel thermophile isolated from the soil exhibits antimicrobial activity against a wide spectrum of bacteria. **Tapas Bhattacharyya**, Supriya Deepak, Rajnikant Sharma, Jitendra Sahoo, Indroneil Ghosh and Ranjana Pathania. Poster presented at 5th Uttarakhand State science and Technology Congress
- Highthroughput screening identifies a small molecule with broad spectrum antibacterial potential. **Tapas Bhattacharyya**, Santosh Kumar Srivastava, Naveen Kumar Navani & Ranjana Pathania. Poster presented at 53rd Annual Conference of Association of Microbiologists of India (AMI) – 2012
- Small molecule screening identifies a novel cell division inhibitor with broad spectrum antibacterial potency. **Tapas Bhattacharyya**, Santosh Kumar Srivastava, Naveen Kumar Navani & Ranjana Pathania. Poster at II International Conference on Antimicrobial Research.

ACKNOWLEDGEMENT

This work is a cumulative effort of many people who have directly or indirectly supported and encouraged me all these years for shaping this work to a success. Without their help it could not be possible. It is an opportunity for me to acknowledge them for their contribution for successful completion of this work.

First of all, I would like to acknowledge my parents for their blessings and unconditional support which gave me the strength to reach at the end of this work. It is their patience, courage and strong belief on me that allowed me to pursue my research interest. I am thankful to my loving sister who always inspired me to attain my research goal.

I would like to owe my gratitude to Dr. Ranjana Pathania, Associate Professor, Department of Biotechnology, IIT Roorkee, my Mentor and Supervisor for guiding me, directing me to right direction and correcting my mistakes. Her admirable scientific pursuit, knowledge and achievements are inspiration for me and encouraged me to take tough scientific challenges in my Ph.D. Her caring and supportive nature always shielded me from difficulties and helped me to overcome many hurdles during my Ph.D. I found myself very lucky to get the opportunity to work under her supervision.

I would like to thank Dr. Naveen Kumar Navani, Associate Professor, Department of Biotechnologt, IIT Roorkee who gave me the opportunity to work with this group. I enjoyed working with him in the NAIP project where he was the PI of the project. His technical suggestions helped me to solve many problems during my Ph.D. He always inspired me to do quality research or to think something innovative. I am really grateful to him for his support.

I would like to thank Prof. Ritu Bartwal, Dr. Partha Roy, Dr. Parvindar Kumar, Dr. A.K.Sharma and Dr. Bijan Chaudhury for extending their support by providing their lab facilities at many occasions.

I am thankful to Prof. G. S. Randhawa, Chairman DRC (Doctoral Research Committee) and Prof. Vikas Pruthi, Chairman SRC (Student Research Committee) for their valuable suggestions and time to time assessment of this work.

I pay my gratitude to Dr. R.K.Peddinti, Associate Professor at Department of Chemistry for giving his valuable time for me whenever it required. I would also like to thank Dr. Naseem Ahmed, Associate Professor at Department of Chemistry for providing his lab facility for me.

I am thankful to Prof. R.P. Sing and Prof. R. Prasad, former Head of the Department of Biotechnology for their unconditional support, encouragement and timely suggestions.

I would like to thank all the respected faculty members in the Department who directly or indirectly helped me, supported me and encouraged me to complete this work.

I would like to acknowledge my previous lab mates Dr. Supriya Deepak Patil, Dr. Rajnikant Sharma, Dr. Santosh Srivastava, Dr. Tarun Sharma, Dr. Jitendra Sahoo, Mrs. Rekha Sharma and Mr. V.Rajesh Iyer for their unconditional supports. It was nice experience of working together with them and leaning many things that helped me to shape up my research work.

Very special thanks go to Dr. Paramesh Ramulu Lambadi and Manasi Gupta who always stood by me and encouraging me to move towards my research goal. Without their help it could not be possible.

I would like to pay a sincere thank to Mr. Pradeep Tarikere Palakshan who was always very cordial and helping me whenever I required in NMR facility.

I am thankful to Dr. Piyush Kalra for extending his support and working together to solve many research problems.

I am quite lucky to have such an enthusiastic batch of people working together with me in the lab. They all are my loving lab mates and always extended their helping hands whenever it required. I would like to mention the name of Atin Sharma, Timsy Bhando, Tamoghna Ghosh, Pardeep Soni, Amit Gaurav, Rajat Dhayani, Jawed Akhtar, Vineet Dubey, Shahnawaz Ahmad Baba and Vandan Sharma . I have good memories with all of them working together, discussing many things during tea break and going for attending a party together. I would like to pay deepest regards to all of them and wish them all a grand success in future.

I would like to thank Anjul Saini for her assistance in ICMR funded projects. Without her support it was really very difficult for me to move ahead in the project.

Many friends and seniors outside the Department also inspired me a lot. I would like to mention Dr. Kuntal Maiti, Dr. Malay Jana, Dr. Chanchal Haldar, Suman Chandra, Chinmoy Kumbhakar and Abhijeet Dhiman who were always very caring and encouraging to me.

I would also like to thank Dr. Ritesh Vijay, Dr. Swapnil Kamle, Mr. Dipak Samal, Mrs. Veena Sardar and Ms. Puja Khobragade for their encouragements.

My sincere thanks to Mohan, Akshay and Ravi for their assistance.

I may forget to mention names of many persons who helped me directly or indirectly in this journey. My sincere apology to them and I would like to express my respect to all of them.

At the end, I would like to thank National Agricultural Innovation Projects (NAIP) and Indian Council of Medical Research (ICMR) for providing funds for this work.

Tapas Bhattacharyya

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C	Degree Celsius
AAC	aminoglycoside acetyltransferase
ABC	ATP binding cassette
Amp	Ampicilin
APH	aminoglycoside phosphoryltransferase
ATCC	American type culture collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumine
CCCP	Carbonylcyanide-3-chlorophenylhydrazone
CFU	Colony forming unit
CIP	Calf intestinal alkaline phosphatase
CLSI	Clinical Laboratory Standards Institute
DAPI	4′, 6-Diamidino-2-phenylindole dihydrochloride
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
DOS	Diversity oriented synthesis
dsDNA	double stranded DNA
EDC	N-(3-dimethylaminopropyl)-N-ethylcarbodiimide
EDTA	Ethylene diamine tetra acetic acid
EPIs	Efflux pump inhibitors
EtBr	Ethidium bromide
FDA	Food and Drug Administration.

g	Grams
GFP	Green fluorescent protein
GNB	Gram negative bacilli
HTS	High throughput screening
h	hours
IDSA	Infectious Disease Society of America
IPTG	Isopropyl thio β, D-galactoside
Kan	Kanamycin
kDa	Kilodalton
L	Liter
LAM	Leeds Acinetobacter Medium
LB	Luria Bertani
MATE	Multidrug and Toxic compound extrusion
MDR	multiple drug resistance
MEC	Minimum effective concentration
MFS	Major facilitator super family
mg	milligram
MH	Mueller Hinton
MH	Muller Hinton
MIC	Minimum inhibitory concentration
min	minutes
ml	milliliter
mM	millimolar
MRSA	Methicillin resistant *Staphylococcus aureus*
NBDs	Nucleotide Binding Domains
ng	Nanogram

Ni-NTA	Nickle Nitrilotriacetic acid
nm	nanometer
nM	nanomolar
NMP	1-(1-naphthylmethyl)-piperazine
OD	Optical density
PAE	Post Antibiotics Effects
PCR	Polymerase chain reaction
PDR	pandrug-resistant
pH	power of hydrogen
QRDRs	Quinolone resistance determining region
RNA	Ribonucleic acid
RND	Resistance Nodulation Division
ROS	Reactive Oxygen Species
rpm	rotation per minute
RpsL	ribosomal binding protein
RT	Room Temperature
s	seconds
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SMR	Small multidrug resistance
TAE	Tris Acetate EDTA buffer
TE	Tris EDTA
TEM	Transmission Electron Microscopy
TEMED	*N*,*N*,*N*,*N* – Tetra methyl ethyl diamine
TKMG	Tris, KCl, $MgCl_2$ and glycerol
TMDs	Trans-membrane domain

t-RNA	yeast transfer RNA’s
UV	ultraviolet
V	Volt
VRE	Vancomycin-resistant enterococci
X- Gal	5–Bromo, 4 – chloro, 3 –indolyl β, D-galactoside
XDR	extensively-drug resistant
µg	microgram
µl	microliter
µM	micromolar

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Emergence of multidrug resistance in various bacterial pathogens has become a huge healthcare concern worldwide. Most of the clinically useful antibiotics are no longer effective against a number of bacterial pathogens. In a report, Infectious Disease Society of America (IDSA) expressed their deep concern regarding the antibiotic resistant bacteria especially "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) and emphasized on the need of new antibiotics to tackle Gram negative bacilli (GNB) resistant to currently available agents. On the other hand, there is a sharp decline in the discovery of new antibiotics since several decades. Most of the clinically useful antibiotics of today were discovered between 1940 and 1960, also known as the golden age of antimicrobial drug discovery. Since then, only 3 to 4 new classes of antibacterial have been introduced. Although infectious diseases are the second leading cause of death worldwide, major pharmaceutical industries have shown disinterest in the investment of antibacterial research because of huge expenditure, time taking process and complicated administrative proceedings. There is an urgent need of new antibacterial molecules with new therapeutic targets as well as finding strategies for rejuvenating the old antibiotics against multidrug resistant pathogens.

Over the years, chemical genetic approach has emerged as one of the most powerful discipline in antibacterial drug discovery. It requires three basic components: a library of chemical collection, an enzymatic or phenotype based assay for screening and a method to identify molecular target of an active molecule. Advent of synthetic chemistry since last few decades has provided a huge resource of diversified collection of small molecules to study the biological systems. Diversity oriented synthesis (DOS) of small molecules along with high-throughput screening technology offers a potentially fruitful means of discovering critically needed new antibacterial agents. Possibility of finding new antibacterial largely depends on the diversity and enormity of chemical collection used for screening. Development of high throughput screening provides us with an opportunity to examine the direct feasible interaction between the diversified chemical resources and the biological specimen. Therefore, phenotype based screening of small molecule library

followed by forward chemical genetic approach can be a practical and effective means of finding novel antibacterial drugs.

For reviving the efficiency of old antibiotics, it is important to understand the root cause of drug resistance and investigate the mechanism by which bacteria exhibit resistance to a particular antibiotic. One of the major causes of multiple drug resistance in bacteria is the presence of transporter proteins (efflux pumps) in their membranes that expel antibiotics from the intracellular environment enabling the bacterial pathogens to survive in presence of antibiotics. Therefore, efflux inhibition can be a viable strategy to counter antibacterial drug resistance. Finding an efflux pump inhibitor against a particular transporter can be an effective means of reviving old antibiotics against these MDR pathogens. Efflux pumps are attractive drug target also because most of them have multiple drug specificity. Efflux pump inhibitors (EPIs) can increase the efficacy of antibiotics and concomitantly decrease the selection of resistant mutants.

Expression of various efflux pumps or transporters in the genome of *Acinetobacter baumannii* is one of the major factors associated with its multiple drug resistance. A number of different families of efflux pumps have been reported in the organism.

Acinetobacter baumannii is one of the most troublesome, opportunistic, Gram negative bacilli associated with multiple kinds of nosocomial infections in human. It is the leading cause of hospital acquired infections worldwide. Till 1970, the organism was susceptible to most of the antibiotics. Over the years it has acquired all the determinants of drug resistance in its genome and transformed itself from a sensitive organism to a completely resistant organism. A mutant of this organism, *Acinetobacter baumannii* AYE is resistant to almost all the antibiotics available in the market. The efflux pumps identified in the organism generally belong to one of the five families, the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family. MATE is the most recently identified multidrug efflux pump family among the other secondary transporters which use electrochemical gradient across the membrane for transporting drugs.

AbeM is a proton driven multidrug efflux pump in *Acinetobacter baumannii* belonging to **MATE** (Multidrug And Toxic Compound Extrusion) family transporter. The efflux pump has diverse substrate specificity and expels many structurally dissimilar substrates including several

clinically useful antibiotics from the cell. Surprisingly, fluoroquinolones are the most common substrates for all the MATE family pumps identified so far in other bacteria also. Therefore, regulatory expression of these pumps might be an important determinant of fluoroquinolone resistance in the organism. Finding an effective efflux pump inhibitor (EPI) of AbeM would result the revival of fluoroquinolones activity against the multidrug resistant *A. baumannii*.

In this study, two different chemical genetic approaches were used to encounter the problem of multiple drug resistance in gram negative bacterial pathogens. Both the approaches utilize small molecule library as a tool for discovering a new chemical entities. The first approach is directed towards the discovery of a new antibacterial molecule against the gram negative bacterial pathogens and the second approach is directed towards discovering a novel efflux pump inhibitor of AbeM, a multidrug efflux pump in *A. baumannii*.

In the first part of the study, a small molecule library of 10956 small molecules has been screened against *E. coli* ATCC25922 in order to find a potential antibacterial molecule against Gram negative bacteria. A whole cell phenotype based screening strategy was adopted for screening the diversified library in liquid media in 96-well plate without any robotic intervention. The screening led to the identification of IITR06144, a 260 Da nitro-aromatic small molecule with excellent broad spectrum antibacterial activity against several multidrug resistant (MDR) Gram negative pathogens. In vitro toxicity studies suggest that the molecule is less toxic than the nitro-aromatic drugs already available in the market, (Nitrofurantoin and Furazolidone). In the study, the probable mode of action of IITR06144 has also been investigated. Study with the knockout and overexpressor mutant of *E. coli* established that NfsA, a major nitroreductase in *E. coli* elevates the potency of IITR06144, once it enters the cell. Microscopic and molecular genetic study has revealed that the molecule causes distortion of FtsZ assembly leading to cellular filamentation. Antisense RNA mediated silencing of FtsZ confirms the interaction of IITR06144 with FtsZ.

In the second part, a library of 8000 small molecules has been screened for finding a potential efflux inhibitor of AbeM in *Acinetobacter baumannii*. AbeM is responsible for transporting number of substrates along with fluoroquinolone antibiotics across the membrane. In *A. baumannii* it confers resistance to fluoroquinolones. The aim of this study is to discover an inhibitor of AbeM and restore the activity of the fluoroquinolones against the resistant mutant of *Acinetobacter baumannii*. *E. coli KAM32* cells exhibit elevated resistance towards the fluoroquinolones when it carries multiple copies of *abeM* cloned into pUC18 plasmid. This phenomenon has been utilized

for screening a small molecule library of 8000 molecules. Screening led to the identification of IITR08027, a small molecule which causes a significant fold of decrease in MIC of ciprofloxacin, norfloxacin and some other non-quinolone substrates against the recombinant *E. coli KAM32*. EtBr accumulation assay confirmed that the molecule enhance the accumulation of EtBr inside the cells. Fluorescence quenching assay on everted membrane vesicles established that the molecule disrupts the H^+ gradient across the membrane and interrupt ciprofloxacin/H^+ antiport. Highly resistant clinical isolate of *Acinetobacter baumannii* (RPTC-19) which express AbeM become susceptible to both ciprofloxacin and norfloxacin in presence of this molecule. Kill kinetic data indicates that 1/4 X MIC ciprofloxacin exhibits its full potency in association with IITR08027. Post antibiotic effect (PAE) of ciprofloxacin was found to be extended in presence of the molecule. Frequency of developing resistant mutant declines in presence of this molecule. IITR08027 exhibited exceedingly low toxicity against the mammalian Hela cells.

CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF NOVEL ANTIBACTERIAL

CHAPTER 2

IDENTIFICATION AND CHARECTERIZATION OF NOVEL ANTIBACTERIAL

2.1. Historical background

Microbes and hosts have a long association. Be it a mutual or a parasitic relationship, microbes play a significant role in the host metabolism either in a positive or negative manner. It took a long time for the world to accept the fact that microbes can cause diseases. It was an era of complete darkness when infectious diseases took millions of lives. Though, the existence of microbes was reported in mid-1600 by Antony van Leeuwenhoek, its role in the disease causing was established after Louis Pasteur disproved the theory of Spontaneous generation and established the Germ theory in mid-1800. The world started to realize that these invisible microscopic entities are responsible for causing diseases in human. This began the journey of antimicrobial chemotherapy. Scientist worldwide started looking for something which can prevent microbes form causing diseases. In 1910, Paul Ehrlich discovered the "magic bullet", salvarsan, the first chemotherapeutic agent, a chemical moiety for the treatment of syphillis. Ehrlich's discovery was an eye opener and the first step towards the modern day antimicrobial chemotherapy. However, an event which revolutionized the entire world was a serendipitous invention by Alexander Fleming. In 1928, he discovered Penicillin, the first successful antibiotic from a natural source. Discovery of Penicillin was a huge impetus to the discovery of new antibiotics from natural sources. The obvious result was the discovery of number of successful antibiotics from natural sources in between 1940 to 1960, a brief period known as "golden age" of antibacterial drug discovery. Suplhonamides, aminoglycosides, chloramphenicol, tetracyclines, microlides and vancomycin were introduced in a very short span of time. In the latter phase, methicillin and nalidixic acid were introduced as synthetic antibiotics.

2.2. Decline in antibacterial drug discovery

Since 1960, there has been a considerable decline in antibacterial drug discovery [1,2]. Although the infectious diseases are second leading cause of death worldwide, major pharmaceutical companies showed disinterest in investment in the antibacterial research because of huge expenditure, time taking process and complicated administrative proceedings. Only a very few antibiotics have been introduced since last 50 years (Fig.2.1). We are largely dependent on old antibiotics which have been discovered earlier. Modified version of several antibiotics with better efficiency have come out in the market, however new discovery was in a

halt for a long time. The antibiotics which can be named after this golden era include linezolid, daptomycin and retapamulin , introduced in 2000,2003 and 2007 respectively. However, these antibiotics belong to the chemical classes oxazolidinones, lipopeptides and pluromutilin which were reported earlier in 1978, 1987 and 1952 respectively [3]. In spite of having good antibacterial potency against resistant pathogens, many drugs like iclaprim, ceftobiplore and oritavancin could not reach the market because of regulatory reasons. Telavacin, an improved derivative of vancomycin has recently been approved by the US Food and Drug Administration (FDA) and reached the clinic to treat methicillin resistant *Staphylococcus aureus (*MRSA) and other gram positive infections [4].

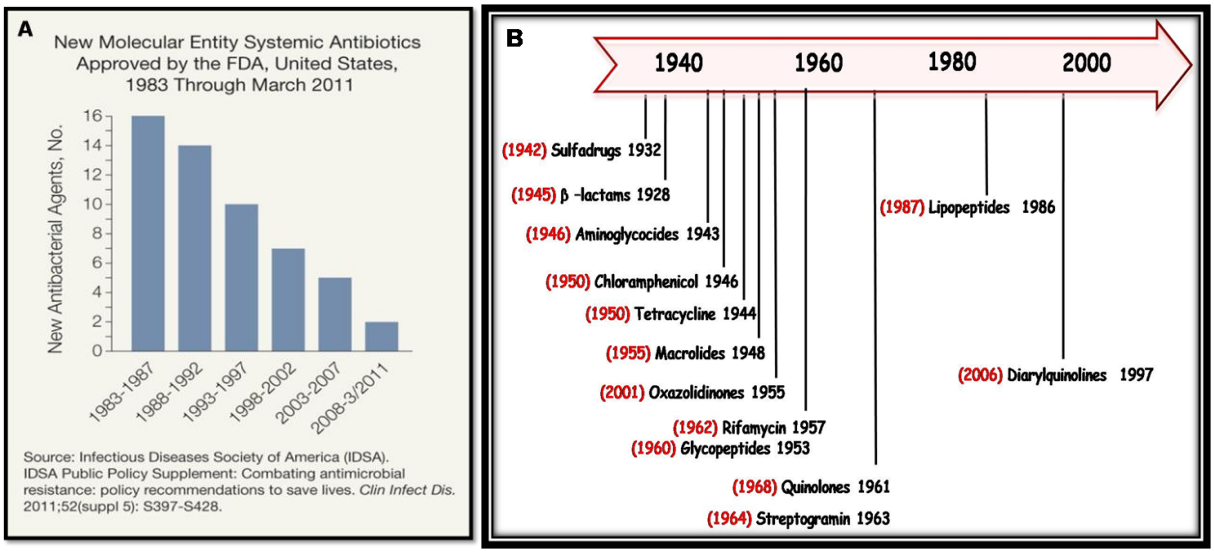


Figure 2.1 (A) Infectious Disease Society of America (IDSA), 2011 report on decline in antimicrobial drug discovery. (Taken from, Combating antimicrobial resistance: Policy recommendation to save lives. Clinical Infectious Disease) (B) Time line of antimicrobial drug discovery. As the new antibiotic discovered, resistant mutant has also emerged indicated with red color (Adapted from Lewis *et al*.2013).

2.3. Emergence of multidrug resistance in bacterial pathogens

With the discovery of new antibiotics, another problem started piling up at an almost equal pace (Fig.2.1B). Extensive and inappropriate use of antibiotics led to the development of drug resistant bacteria. The emergence of multiple drug resistance (MDR) in bacterial pathogens has become a serious healthcare concern worldwide. New terms like extensively-drug resistant (XDR) and pandrug-resistant (PDR) have been introduced to explain the extent of drug resistance. Within a few years of discovery, resistance phenomenon has developed against almost all the drugs. As a result, existing "old" antibiotics are facing serious challenges [5]. In a report, Infectious Disease Society of America (IDSA) expressed their concern regarding the

antibiotic resistant bacteria especially "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) and emphasized on the need of new antibiotics to tackle Gram negative bacilli (GNB) resistant to currently available agents [6]. *A. baumannii*, a gram negative bacillus has become the major cause of hospital acquired infection worldwide by acquiring resistance to most of the antibiotics[7]. Similarly , Methicillin resistant *S. aureus* has been established as a leading cause of nosocomial infections worldwide [8]. *P. aeruginosa* is also a major threat among the other hospital acquired infections because of its high intrinsic resistance to many antibiotics and its ability to develop resistance to multiple classes of antibiotics very rapidly [9]. Vancomycin-resistant enterococci (VRE) generally attack immunocompromised individuals particularly in hospital settings and no specific antimicrobial treatment is available for it [10]. Increasing frequency of panresistant carbapenemase-producing *K. pneumoniae* infections limits the choice of antibiotics for its treatment [11-13].

2.4. Causes of multiple drug resistance

Bacteria have multiple means of acquiring drug resistance. Major cause of acquiring resistance is horizontal transfer of drug-resistant genes among the neighbouring species via transfer of plasmids, transposons or integrons. There are several biochemical mechanisms by which bacteria exhibit resistance to particular antibiotics.

2.4.1. *Alteration in target protein*

Many bacteria alter the target proteins in such a manner that the drug molecule cannot bind to them. This is achieved by acquiring mutation in the coding region of the target protein. Fluoroquinolone resistance in many bacteria is due to the mutation and structural modification of topoisomerase. Many laboratory *E. coli* strains exhibit increased susceptibility to streptomycin due to the mutation in ribosomal binding protein (RpsL). Mycobacterium strains exhibit resistance to aminoglycosides because of the mutation in ribosome binding site [14].

2.4.2. *Enzymatic inactivation of drug molecule*

Many bacteria adapt the mechanism of altering the drug by an enzymatic inactivation. β-lactamase producing bacteria exhibit resistance to β lactam antibiotics by enzymatic hydrolysis. Many aminoglycosides are inactivated by aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC), aminoglycoside adenyltransferase or

nucleotidyltransferase. These enzymes modify the drug by phosphorylation, acetylation and adenylation respectively.

2.4.3. *Efflux pumps*

Another major cause of multidrug resistance in bacteria is the presence of efflux pumps or transporter proteins in their membrane which pumps out the antibiotics from the intracellular environment and enable the bacteria to survive in the presence of antibiotics. Number of efflux pump transporter proteins have been identified and characterized in many drug resistant pathogens. They are transmembrane proteins having one outer membrane domain, a cytoplasmic inner membrane domain and intra membrane domain. Clinically relevant transporters are broadly classified into five major categories. Four of these members belong to the secondary transporter as they are driven by the electrochemical gradient across the membrane. Secondary transporters are primarily responsible for rendering MDR (multiple drug resistance) to bacteria. These secondary efflux pumps include Major facilitator super family (MFS), Resistance nodulation and cell division (RND), Small multidrug resistance (SMR) and Multidrug and Toxic compound extrusion (MATE). The fifth member of the efflux pump, ATP binding cassette (ABC) transporter is a primary transporter driven by the ATP [15, 16].

2.5. Approaches in drug discovery

Numerous approaches have been adopted by various research groups worldwide to find new antibacterial agents from natural as well as synthetic sources. The emphasis is always on finding a new therapeutic target which has not been explored yet. The probability of developing drug resistance for a new target is always lesser in comparison to a common target. There are some alternative strategies which look for a chemical moiety capable of augmenting or reviving the activity of an inactive old antibiotic. Combination of antibiotic and nonantibiotic are sometime very useful for reviving the activity of certain antibiotic [17]. Finding an efflux pump inhibitor against a particular pump is equally effective to revive the activity of certain antibiotic against multiple drug resistant mutants [18].

2.6. Screening for novel antibacterial

Any drug discovery program begins with the screening of diversified chemical collection. It can be a systematic collection of synthetic molecules or natural products from numerous sources. There can be two basic approaches of screening: target based and

phenotype based. In target based screening a metabolic pathway is targeted. One of the crucial steps in the pathway is blocked by inhibiting a particular enzyme. An assay is developed on the basis of the biochemical reaction of that particular step. In phenotype based screening whole cells are monitored before and after the exposure of chemical collection. If any observable change in the phenotype occurs due to an active chemical moiety, next step is to find the target gene or protein by using bimolecular techniques [19, 20]. For validating novel target, new approaches are needed that facilitate small molecule screening efforts on large numbers of incompletely characterized targets [21, 22].

2.7. Combinatorial synthesis and high throughput screening

Due to the advent of synthetic chemistry since last few decades, combinatorial synthesis has provided a huge resource of diversified collection of small molecules to study the biological system [23]. Diversity oriented synthesis (DOS) of small molecules offers a potentially fruitful means of discovering critically needed new antibacterial agents [24]. High throughput screening (HTS) fuels the drug discovery pipelines of the majority of pharmaceutical companies [25] as well as academia [26] and is one of the central paradigms of modern drug discovery . However, establishing a high-throughput facility in an academic framework require investments and strategies which are very uncommon to the academic researchers [27]. The aim of high throughput drug discovery is to test large compound collections for potentially active compounds (‘hits’) in order to allow further development of compounds for pre-clinical testing (‘leads’) [28]. Diversified chemical resources accompanied by high-throughput screening technology and many other molecular genetics techniques together can be a means of developing new antibacterial drugs with novel modes of action [29]. There are many early and recent reports of bioactive small molecules with different molecular targets [30-39]. These success stories definitely encourage the researchers to focus on the chemical genetic approaches in antibacterial drug discovery.

2.8. Target identification

Identification of a molecular target of a lead molecule always remains to be the major hurdle of any drug discovery projects [40]. However, numerous approaches have been adopted so far to validate the bio-molecular target. PCR based ordered genomic libraries [41] , gene expression profiling [42], multi copy suppression [35,43], antibiotic sensitivity profiling of knockout mutant [44], antisense expression of target genes [45,46] and use of high frequency transposition [47] are the few approaches which have been utilized for target identification.

Bacterial reporter system has also been employed for monitoring a specific cellular response due to the drug treatment [48,49]. Any of these approaches or combination of these can be fruitful means of validating the biological target of a small molecule. Antibiotics as well as small molecules can be used as a valuable tools for understanding the complex bacterial physiology [50].

2.9. Bacterial survival mechanism in stress

Antibiotics are known to give rise to stress like phenomenon in bacterial cells [51]. Bacteria have a complex mechanism of survival against stress condition. Filamentation has been implicated as one of most common survival mechanism in bacteria as an innate defense during the stress conditions [52]. There is much evidence which indicate that the filamentation occurs due to the inhibition of septation during cell division [52,53]. FtsZ is the major protein responsible for septum formation in the mid-cell region during cell division and can be inhibited either by SulA mediated [22,54] or SulA independent pathway[55] during the SOS response. SulA, a major partner of SOS cascade proteins, is activated due to auto-proteolysis of LexA repressor by the influence of RecA and inhibits FtsZ polymerization during septum formation [52,55]. Inhibition of FtsZ may be accomplished by SlmA, nucleoid occlusion factor, a protein which does not allow the polymerization of FtsZ until the chromosome is segregated properly during cell division [56,57]. MinCD may also block the septation by inhibiting FtsZ polymerization leading to cell filamentation [58]. SulA, MinCD and SlmA are major internal players that interact with FtsZ assembly during bacterial cell division.

2.10. Bacterial cell division and FtsZ assembly

Bacterial cell division process is broadly divided in two fundamental events; chromosomal segregation followed by cytokinesis. Once the replication of DNA is over and the genetic material is ready to be segregated, midcell region of the cell involves in aggregating the necessary protein family members of cell division (Fig.2.2). Fts are the key protein family involved in the processes which together form a divisome ring also called Z-ring at the separating region of two dividing cells. At least ten different members of these Fts family proteins are recruited sequentially in the process as the cell division progresses [59]. FtsZ is the key member of the system which gets polymerized to form the Z-ring. ZipA, an inner membrane anchoring protein in the assembly initiates the bundling of FtsZ by interacting with its C-terminal end [60]. The other important proteins in the groups are FtsA, ZipA, FtsK, FtsB, FtsQ, FtsN, FtsW, FtsI and AmiC. Role of nitroheterocyclic compounds on bacterial cell

division and their interaction with FtsZ assembly is very unclear. The reduced form of these molecules not only target DNA but also attack multiple cellular proteins [61].

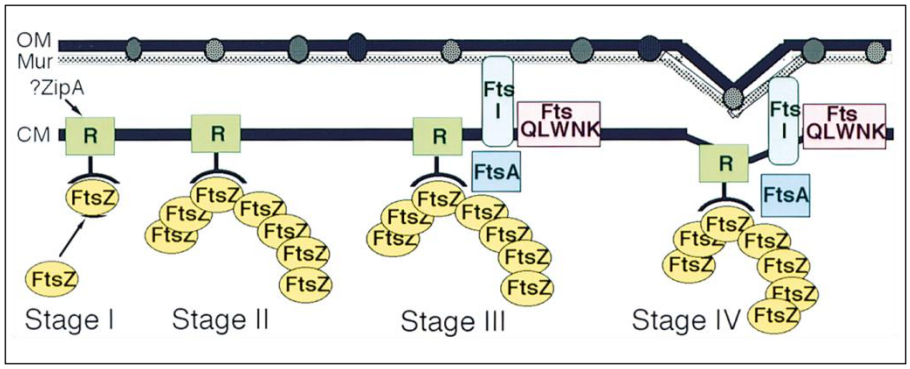


Figure 2.2 Different stages of forming FtsZ assembly for Z-ring formation (Image from, Rothfield *et al*. 1997) [*Permission taken. Please see the permission letter at the end of this chapter*]

2.11. Nitroheterocyclic compounds and their therapeutic target

Nitroheterocyclic compounds have been using as chemotherapeutic agents for a long time. Nitro group is essential for the therapeutic activity of these compounds [62]. In bacteria, nitro heterocyclic compounds are reduced by flavoprotein NAD(P)H linked reductases. The reduced form of nitroheterocyclic compounds interact with DNA and cause a single strand break [63, 64]. In *E. coli* two types of nitroreductase activity have been reported: Oxygen insensitive nitroreductase and oxygen sensitive nitroreductase. *nfsA* and *nfsB genes* encode proteins of oxygen insensitive reductase system [65-67]. Both of these enzymes are responsible for reducing the nitro group of the nitroheterocyclic compounds in a stepwise manner leading to the generation of various reactive intermediates which cause DNA damage [68,69]. However, the exact molecular mechanism and target of nitroheterocyclic compounds remain very unclear.

In this study, a novel nitroheterocyclic molecule with broad spectrum antibacterial potency has been identified after screening a small molecule library of 10956 molecules. The interaction of the molecule with bacterial cell division proteins were investigated by using chemical genetic techniques.

2.12. Identification and characterization of a novel antibacterial molecule from a small molecule library

The following section of this chapter deals with detailed description of the study which was carried out for identification and characterization of novel antibacterial molecule against gram negative bacteria.

2.12.1. Primary screening of small molecule library

This study began with phenotype based whole cell screening of a small molecule library consisting of 10956 compounds against *Escherichia coli* ATCC25922. The objective was to discover novel antibacterial small molecules against Gram negative bacteria.

The small molecule library which has been used in this study was purchased from Maybridge (Trevillett, UK). It consists of a diversified collection of 10956 drug-like small molecules. The average molecular weight of the compounds in the library ranges between 100 to 600 Da (Fig.2.3). The compounds are mostly aromatic, nitro aromatic or halo-aromatic in nature. These are individually designed compounds, produced by innovative synthetic techniques, based on heterocyclic chemistry.

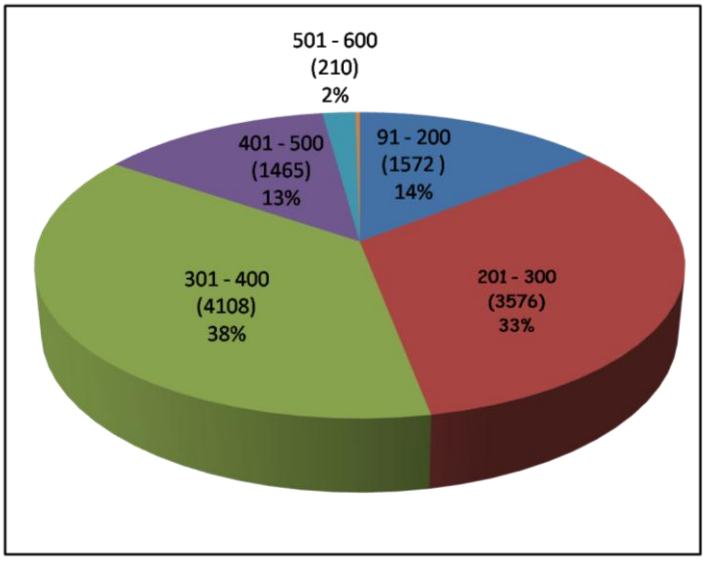


Figure 2.3 Molecular weight ranges of the small molecule library.

Before the start of any screening program, it is important to archive the compounds of the library in an order in the 96 well format so that they can be screened in a systematic order in a high-throughput fashion. Archiving is defined as giving every compound a physical address in the 96 well plates. A total of 10956 compounds were arranged in 141- 96-well plates. Each plate contains 80 compounds as similar arrangement in the first plate (Fig.2.4). The wells at the first and last columns of the 96-well storage plate were kept blank. No molecules were stored in those wells. During the primary screening, these wells were assigned for controls in the assay. Position of each molecule in a particular plate is fixed. Each compound was given a five digit IITR identification number. The compound no 1 was designated as IITR00001 and similarly the compound no 10956 was assigned as IITR10956.

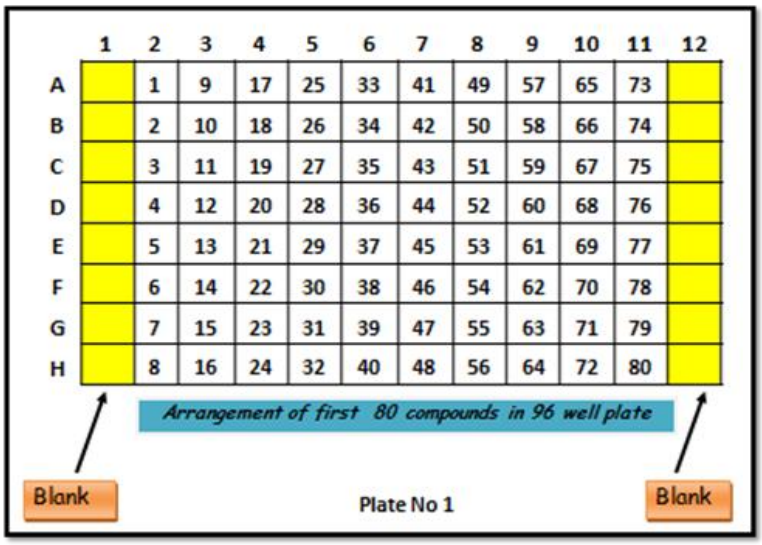


Figure 2.4 Arrangement of 80 different small molecules in plate no 1.

The first step of any screening process is to dissolve the powdered compounds in a suitable solvent to prepare the primary stock of a desired concentration - a process known as solubilization that makes samples readily available for biological testing. Dimethyl suphoxide (DMSO) is the most common solvent in compound solubilization because of its physicochemical properties, high solvent power, low chemical reactivity, and low toxicity. DMSO (Merck, Germany) was used for dissolving the compounds. All 10956 compounds were dissolved individually in different volumes (according to their molecular weight) in 100% pure DMSO to get the final concentration of 50 mM (Primary stock). The primary stock solutions were distributed individually in 141-96-well plates as they were archived previously. The primary stocks were diluted five folds in same arrangement in different 96 well plates by using multichannel pipette to get the first intermediary stock of 10 mM. First intermediary stocks were again diluted in a same manner to get secondary intermediary stock of 2.5 mM (Fig.2.5).

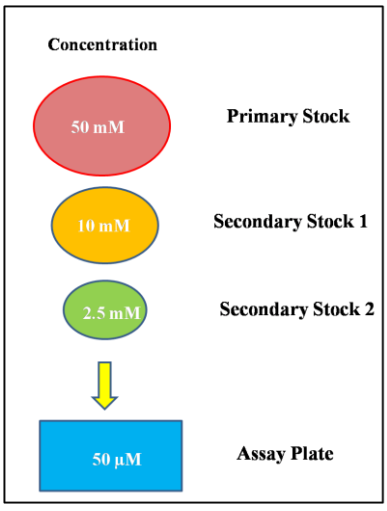


Figure 2.5 Different stocks that have been prepared for the Maybridge small molecule library.

2.12.2. Determining the sensitivity of DMSO against *Escherichia coli* ATCC25922 (Indicator strain)

2.12.2.1. Materials

Mueller Hinton (MH) broth (Merck, Germany), 96-well polystyrene flat bottom plates (Axygen), 96 well plate reader (SpectraMax M2e Plus, Molecular Devices, USA), Multichannel pipettes (Eppendorf, Germany).

2.12.2.2. Methods

2.12.2.2.1. Media preparation

25g Mueller Hinton (MH) broth powder was dissolved in 100 ml of distilled water. Final volume was adjusted to 1000 ml with distilled water and was autoclaved for 15 min at 121°C (15 lb/in^2).

2.12.2.2.2. Inoculum preparation of the indicator strain (E. coli ATCC25922)

Freshly streaked single isolated colony from a fresh MH agar plate culture of *E .coli* ATCC25922 was inoculated into a culture tube containing 5 ml of MH broth medium. The broth culture was incubated at 37°C, 250 rpm for 12 h. This culture was then subcultured (1%) into fresh 5 ml MH broth medium tubes and incubated at 37°C, 250 rpm until the culture reached OD between 0.6 – 0.7 at 600 nm. The cells were diluted 10^3 times in the MH broth media for the assay in 96-well plate.

2.12.2.2.3. Preparing different percentage of DMSO

Sterile MH broth of 500 µl with different percentages (1-20%) of DMSO was prepared inside the laminar hood in different sterile micro-centrifuge tubes (1 ml capacity). 100 µl of each stock was placed in a sterile flat bottom 96-well plates in triplicate. Three wells were kept with only sterile MH broth as appositive control.

2.12.2.2.4. Sensitivity of different percentages of DMSO against E. coli ATCC25922

100 µl of the freshly prepared inoculums as described earlier in section 2.12.2.2.2 was added to each triplicate wells by a multichannel pipette. A pre-incubation OD at 600 nm was measured by a plate reader. The 96 well plate was incubated at 37°C in a static condition inside a humidity controlled incubator with 85% humidity for 12 h and absorbance was measured at 600 nm using SpectraMax M2e Plus (Molecular Devices) plate reader.

2.12.2.3. Result

It was observed that at 2% DMSO, the growth of the indicator strain was very little affected (Fig.2.6). Therefore, primary screening was done at 2% DMSO and a DMSO control was kept in the assay plates which do not contain any drug. The percent growth in 2% DMSO was considered as maximum (100%) growth in the primary screening assay. This was referred to as high control. The growth in the presence of test compound was determined in comparison to the high control with 2% DMSO.

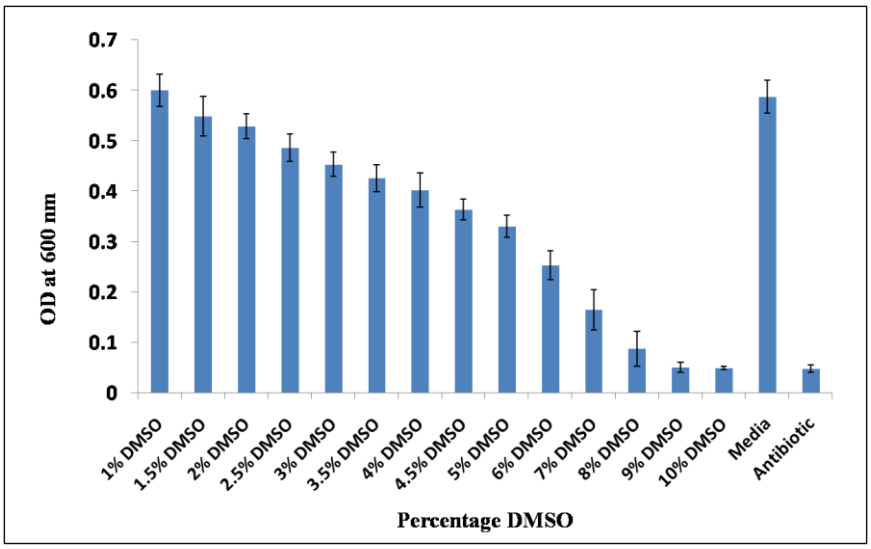


Figure 2.6 Sensitivity of *Escherichia coli* ATCC25922 against different percentage of DMSO.

2.12.3. Optimization of growth condition of *E. coli* ATCC25922 for primary screening

Primary screening of the chemical library has been done against gram negative *Escherichia coli* ATCC25922 as an indicator strain. Growth profile of the indicator strain was optimized for the particular assay condition. The objective was to use healthy cells from logarithmic phase for the assay.

2.12.3.1. Materials

Mueller Hinton (MH) broth (Merck, Germany), Spectrophotometer (SpectraMax M2e Plus, Molecular Devices, USA), culture tubes (50 ml capacity) and conical flask (250 ml capacity.

2.12.3.2. Method

A freshly streaked single colony was picked from the surface of fresh MH agar plate and inoculated into sterile 5 ml of MH broth in a culture tube and incubated at 37ºC, 250 rpm in a

humidity controlled shaker incubator. Overnight grown culture was subcultured 1% (v/v) in 250 ml flask containing 100 ml of sterile MH broth.

2.12.3.3. Result

It was observed that after 3 h of incubation at 37°C with 200 rpm agitation, the absorbance of the culture reaches at 0.67 to 0.7 at 600 nm (Fig.2.7). The culture was diluted 1000 fold in sterile MH broth and 100 µl of this culture was used during the primary screening assay.

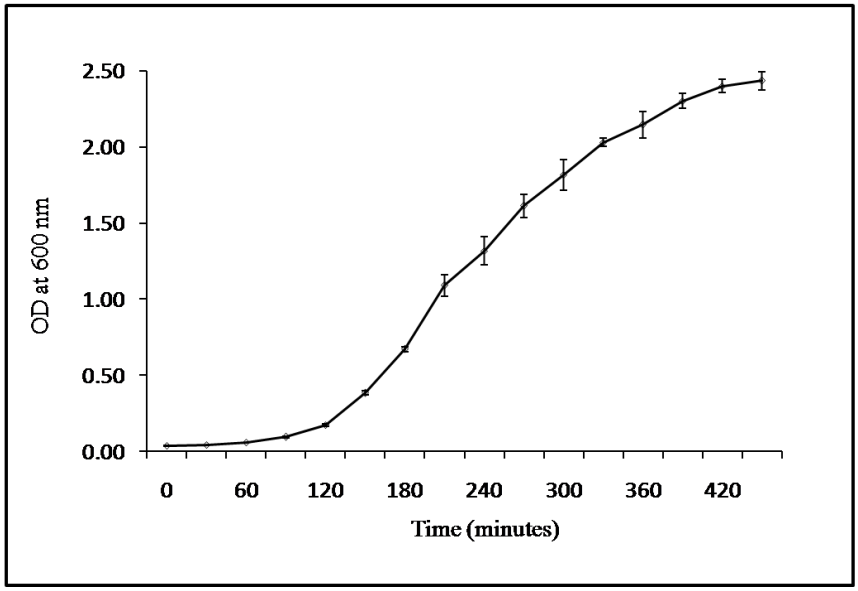


Figure 2.7 Growth kinetics of *Escherichia coli* ATCC25922 (indicator strain).

2.12.4. Primary Screening Assay

The assay for primary screening was designed in such a manner that all the compounds can be screened in a high throughput fashion in duplicate (Fig.2.8 and Fig.2.9). Total 80 compounds were screened at a time in two replica plates. There were four controls in the assay. These were high control with 2% DMSO, high control with no DMSO, antibiotic control (low control) and a media control. The high control with 2% DMSO was used as a positive control having the maximum possible growth. Antibiotic control having 50µM kanamycin was used as a negative control with minimum growth. The percent growth in the test wells were calculated in terms of the high control with 2% DMSO because all the test well also contain 2% DMSO as the compounds were dissolved in 100% pure DMSO. The media control was used to confirm that the media in the assay is not contaminated. Media controls do not contain any inoculum. The high control without the DMSO was used to monitor that the growth during the assay not inhibited by other external factors.

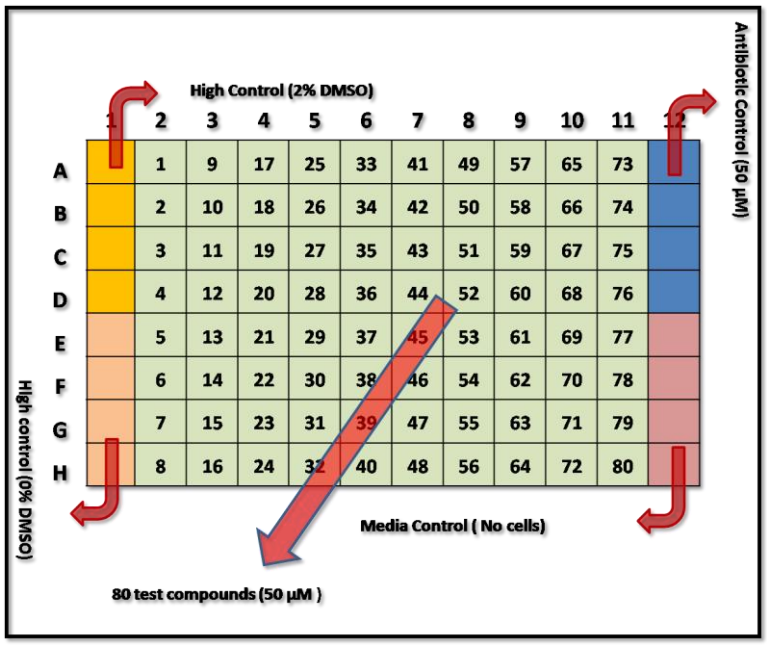


Figure 2.8 Arrangement of assay plate for primary screening.

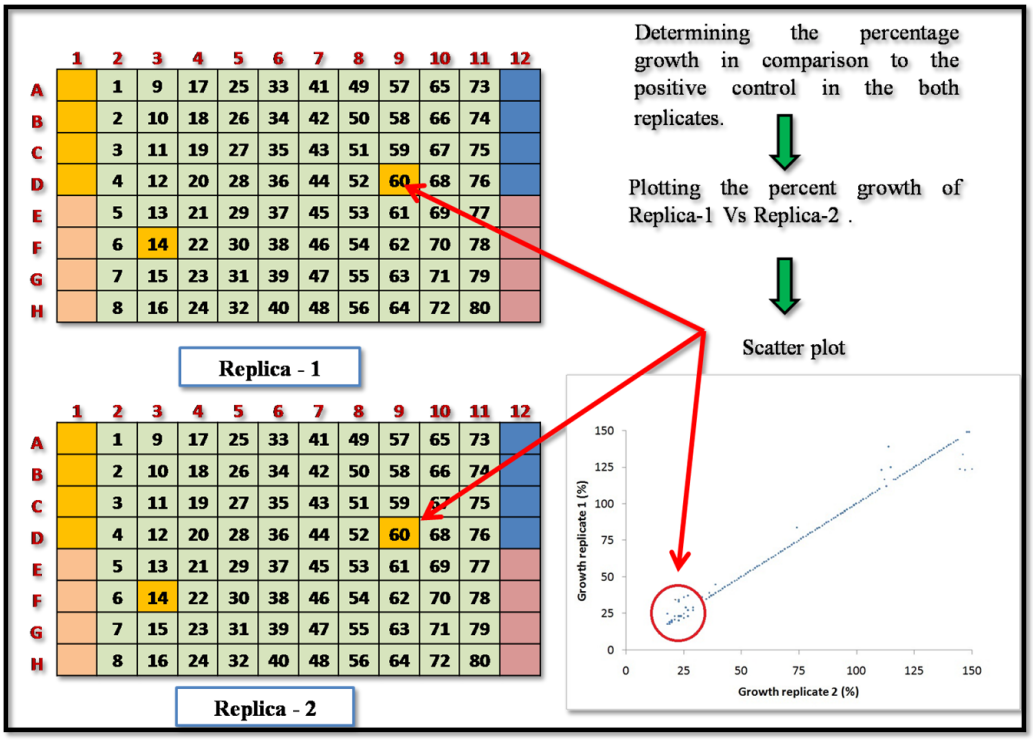


Figure 2.9 A schematic diagram of primary screening assay.

All 10956 compounds were tested in duplicate at a concentration of 50 µM against *E. coli* ATCC 25922. The entire screening was carried out in 16 different batches (Fig.2.10-2.18). 800 molecules were screened at a time except the last batch which consisted of 560 molecules. All the assays were set up in aseptic condition inside a laminar hood. The bacterial growth was measured in terms of measuring the absorbance at 600 nm by using a plate reader (SpectraMax

M2e Plus, Molecular Device). The changes in OD after 12 h of incubation were considered as absolute growth. The percent growth was calculated in comparison to positive control with 2% DMSO. Following formula was used to calculate the percentage growth

Percentage Growth = (OD_{600} in the test well / OD_{600} in the Positive solvent control) x 100

For each molecule, two values of percentage growth were observed which have been plotted in scatter plot along the X and Y axis.

2.12.4.1. Materials

Mueller Hinton (MH) broth (Merck, Germany), Small molecule library in 96-well plates, Kanamycin, 96-well polystyrene flat bottom plates (Axygen), culture tubes (50 ml capacity) and conical flask (250 ml capacity), Multichannel pipettes (Eppendorf, Germany), plate reader (SpectraMax M2e Plus, Molecular Devices, USA), Humidity controlled incubator and biosafety cabinet.

2.12.4.2. Methods

2.12.4.2.1. Inoculum preparation

Inoculum was prepared as described in previous section 2.12.2.2.2. *E. coli* ATCC 25922 cells of an OD_{600} of 0.7 were diluted 1000 fold in sterile MH broth and 100 µl of this culture was used during the primary screening assay.

2.12.4.2.2. Preparing 96-well plates for the primary screening assay

1. 96 µl of sterile MH broth was dispensed into all the 96 wells of the assay plate by using a multichannel pipette.
2. 4 µl of the small molecules from the stock of 2.5 mM was transferred to 80 different wells (test wells) as indicated in Fig.2.8.
3. 4 µl of DMSO was transferred to each well of A1, B1, C1 and D1 (positive solvent control wells).
4. 4 µl of sterile media (MH broth) was transferred to each wells of E1, F1,G1 and H1(positive control wells)
5. 4 µl of antibiotic (kanamycin) from 2.5 mM stock was transferred to each wells of A12, B12, C12 and D12 (negative control/ antibiotic control wells)
6. 104 µl of sterile MH broth was transferred to each well of E12, F12, G12 and H12. The total volume in these wells was 200 µl (media control wells).

7. 100 µl of freshly prepared inoculums was poured into all the wells excepting E12, F12, G12 and H12.
8. A pre-read of the plate at OD 600 nm was measured by using a plate reader for preread of the plate.
9. The plates were then incubated at 37°C in a static condition inside a humidity controlled incubator for 12 h.
10. OD_{600} after 12 h incubation (post-read) was measured by using the Spectramax M2e Plus plate reader at 600 nm.
11. Difference in OD before and after incubation was calculated by subtracting the pre-read values from the post-read values.
12. The average OD at A1, B1, C1 and D1 was calculated.
13. Percentage OD at every well was calculated in comparison to the average OD_{600} of A1, B1, C1 and D1.
14. For every molecules, two growth percentage values were found in two plates (Plate-1 and Plate-2) as indicated in the Figure.
15. A scatter plot was drawn where two growth percentage values were used as X and Y coordinates respectively.
16. The molecules which resulted in less than 25% growth in both the plates were selected as primary hits.

2.12.4.3. Result

Scatter plots of primary screening in 16 batches are given in Fig.2.10- 2.17. Scatter plot of all 10956 molecules are given in Fig.2.18.

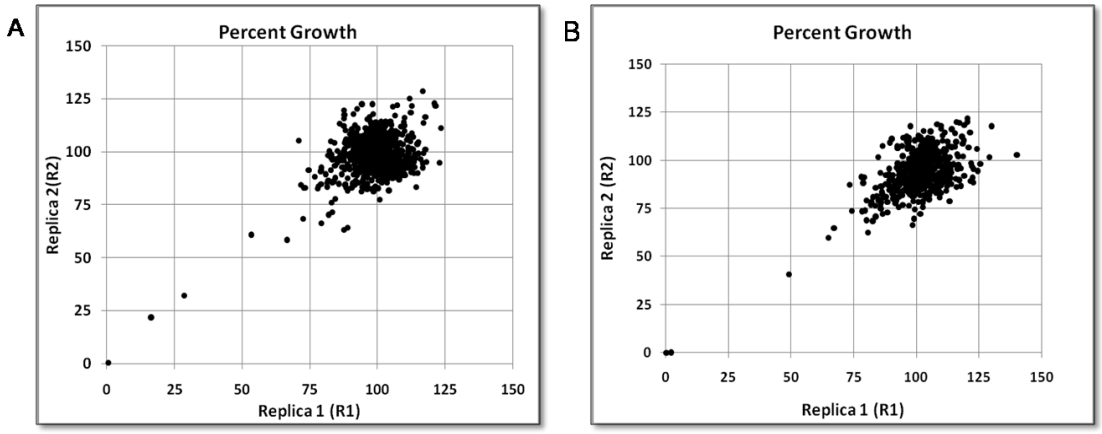


Figure 2.10 Primary screening of (A) IITR00001 to IITR00800 (B) IITR00801 to IITR01400 against *E. coli* ATCC25922.

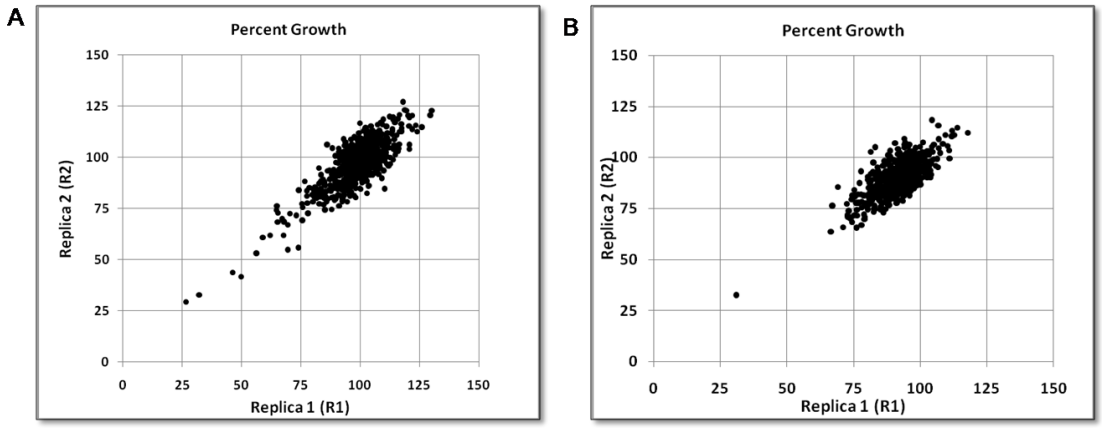


Figure 2.11 Primary screening of (A) IITR01401 to IITR02200 (B) IITR02200 to IITR02800 against *E. coli* ATCC25922.

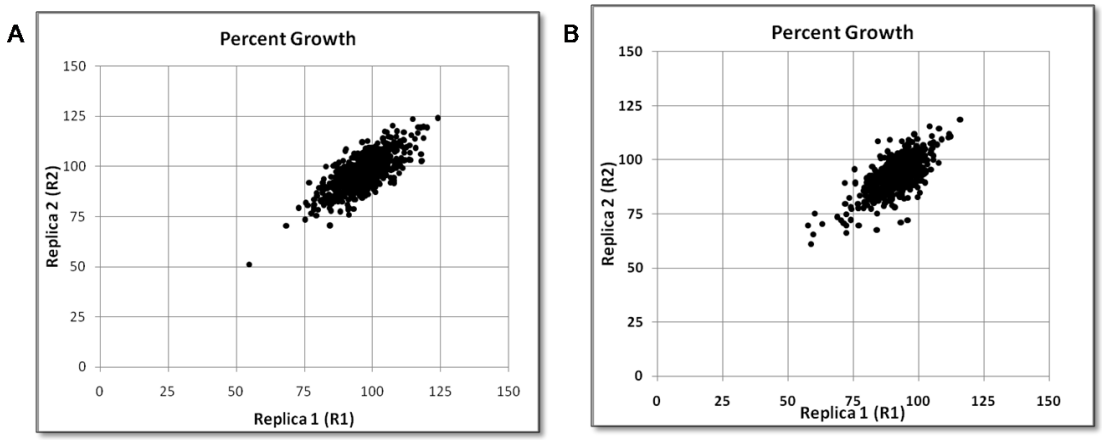


Figure 2.12 Primary screening of (A) IITR028001 to IITR03600 (B) IITR03601 to IITR04200 against *E. coli* ATCC25922.

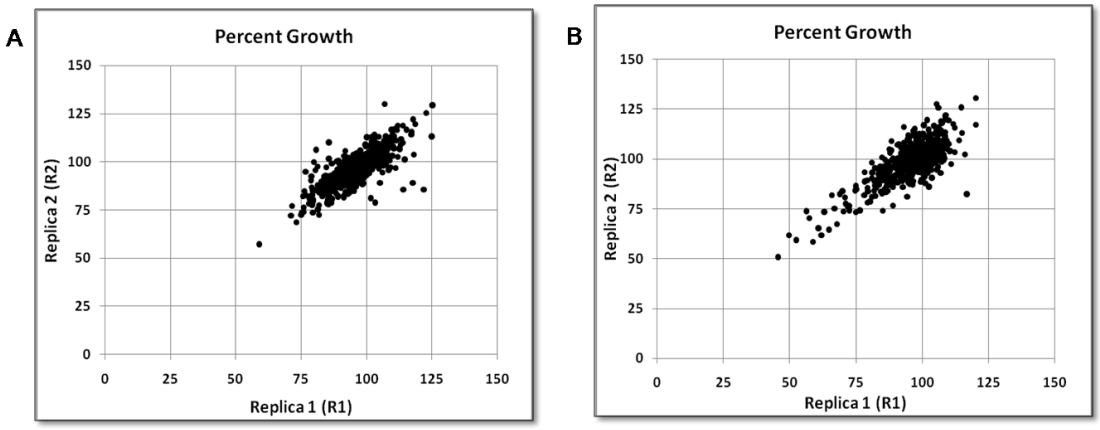


Figure 2.13 Primary screening of (A) IITR04201 to IITR05000 (B) IITR05001 to IITR05600 against *E. coli* ATCC25922.

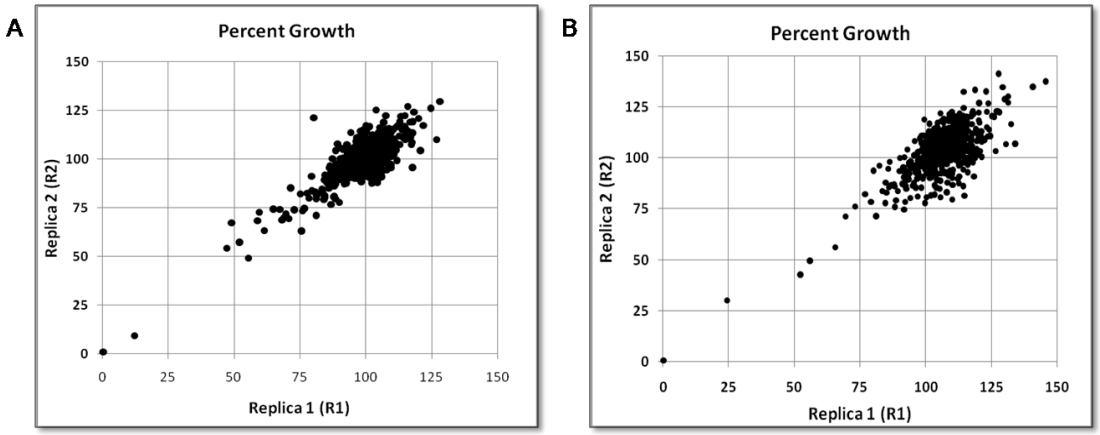


Figure 2.14 Primary screening of (A) IITR05601 to IITR06400 (B) IITR06401 to IITR07000 against *E. coli* ATCC25922.

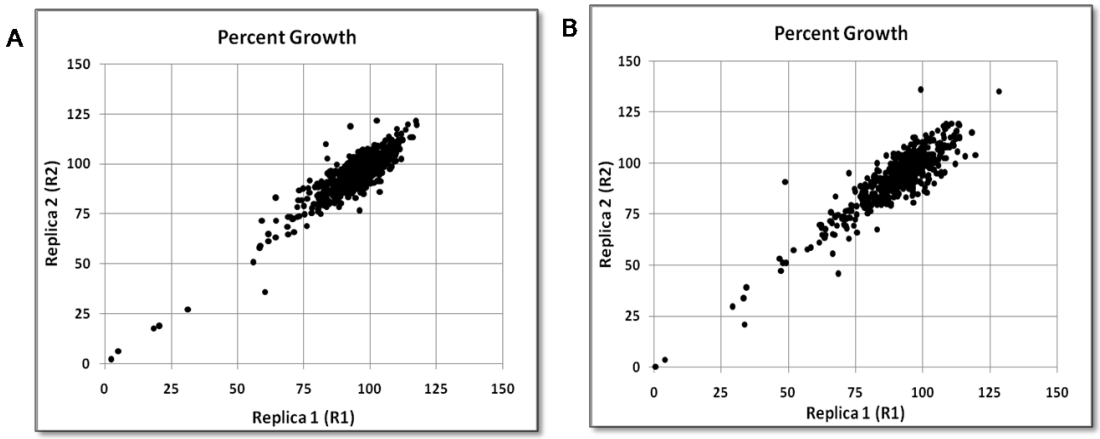


Figure 2.15 Primary screening of (A) IITR07001 to IITR07800 (B) IITR07800 to IITR08400 against *E. coli* ATCC25922.

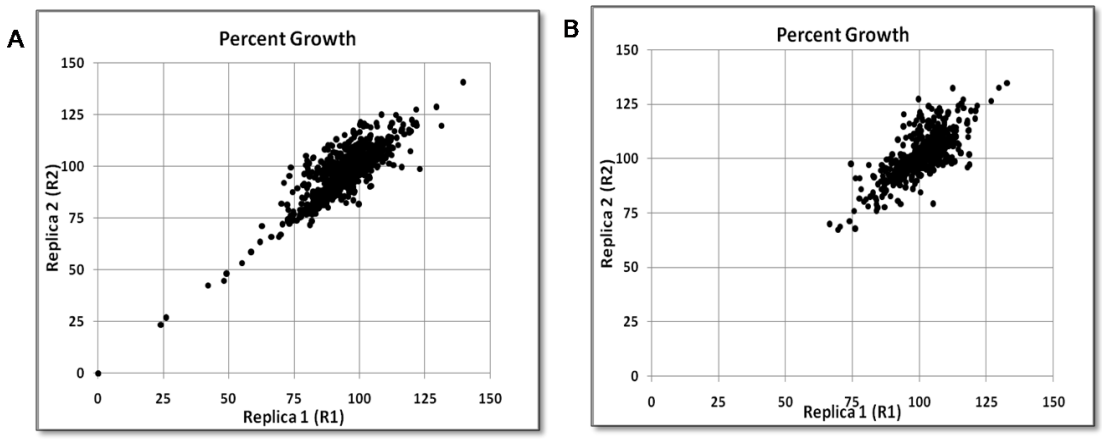


Figure 2.16 Primary screening of (A) IITR08401 to IITR09200 (B) IITR09200 to IITR09800 against *E. coli* ATCC25922.

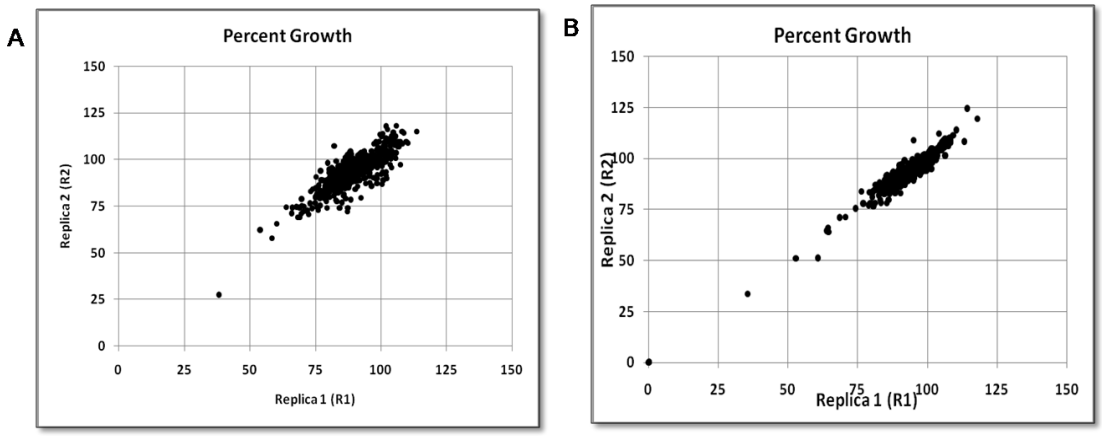


Figure 2.17 Primary screening of (A) IITR09801 to IITR10440 (B) IITR10440 to IITR10956 against *E. coli* ATCC25922.

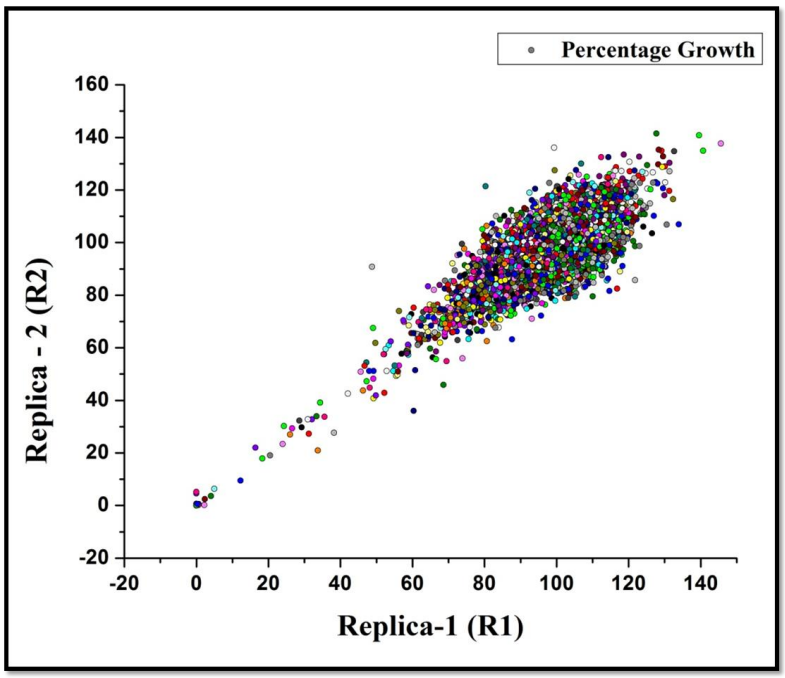


Figure 2.18 Primary screening of all 10956 molecules against *E. coli* ATCC25922 (combined).

Primary screening identified 223 compounds that displayed good antibacterial activity against *E. coli* ATCC25922 (Fig.2.19). Out of 223 primary hits, 30 compounds displayed excellent activity inhibiting 75% of growth in comparison to the positive control in both the replica.

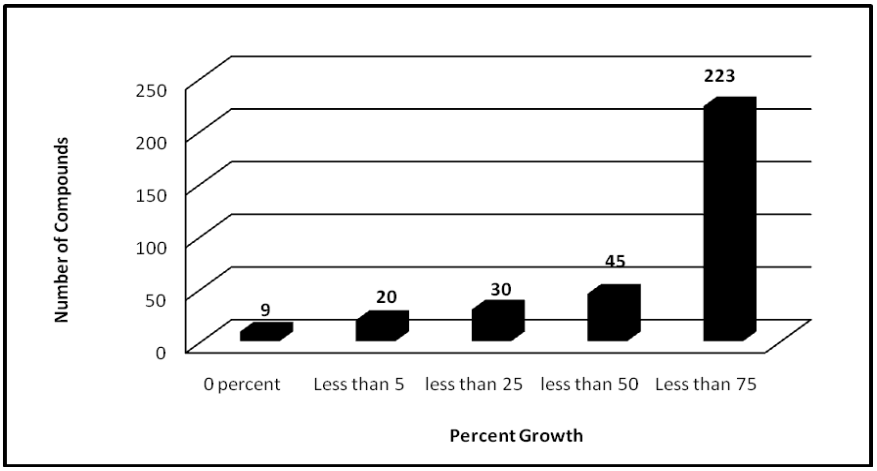


Figure 2.19 The number of antibacterial small molecule identified after screening the library in terms of different percentage of growth observed against *E. coli* ATCC25922.

Flow diagram of screening strategy

Small molecule library (10956 compounds)

⇩ Archiving

Primary stock

Dissolve it in respective volume of 100% pure DMSO (50 mM stock)

⇩

Secondary stock (10 mM) in replica plate

⇩

Secondary stock (2.5 mM) in replica plate

⇩

Assay plate (50 µM) in replica plate

⇩

Primary screening against *Escherichia coli* ATCC 25922 in duplicate

⇩

223 Primary hits

(Growth inhibitory compounds)

⇩

30 leads

(Most effective compounds)

⇩

Determining MIC against Gram negative, gram positive and multidrug resistant pathogens and future studies

2.13. Secondary screening against broad range of pathogenic bacteria

30 lead molecules identified in primary screening were tested against a spectrum of Gram negative, Gram positive and multidrug resistant clinical isolates at secondary level.

2.13.1. Determining the MIC of antibacterial small molecules

The MIC of 30 lead molecules were determined against all these strains by two fold broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines in 96 well plates in Muller Hinton broth.

2.13.1.1. Materials

Mueller Hinton (MH) broth (Merck, Germany), 96-well polystyrene flat bottom plates, 96 well plate reader and multichannel pipette.

2.13.1.2. Methods

2.13.1.2.1. Inoculum preparation of the test organism

A single colony of the test organism was picked from a freshly streaked agar plate and inoculated into a culture tube containing 5 ml of MH Broth. The broth culture was incubated at 37°C, 250 rpm for 12 h. This cells were then subcultured (1% v/v) into fresh 5 ml MH broth medium tubes and incubated at 37°C, 250 rpm until the culture reached OD between 0.6 – 0.7 at 600 nm. The cells were diluted 10^4 times in the MH broth media for the assay in 96-well plate.

2.13.1.2.2. Assay set up in 96-well plate

1. The serial dilutions in 96- well plate was carried out in duplicates for each antibacterial molecule to be tested.
2. 196 µl of sterile MH broth was added to the 12^{th} well of the 96 well plate (A12-H12). 4 µl of antibacterial molecule from 50 mM primary stock was added to make final volume of 200 µl.
3. 100 µl of the MH Broth was added in the remaining wells (except for wells A12-H12).
4. For serial dilution, 100 µl supernatant from 12^{th} well was transferred to 11^{th} well. The serial dilution continues similarly up to 3^{rd} well.
5. 100 µl of the contents from 3^{rd} well is transferred directly to the 1^{st} well (as 2^{nd} well is used as positive control i.e. without bacterial extract).

6. 100 µl of the diluted (10^{-4}) test strain was added to all the wells except 1st column wells (A1-H1) that served as negative control.
7. The final volume in all the wells was 200 µl.
8. The pre-incubation reading was recorded with the help of a plate reader at 600 nm.
9. The plate was incubated for 12 h at 37°C in a humidity controlled incubator.
10. The post- incubation reading was taken after 12 h of incubation.
11. The change in OD of each well is calculated by subtracting the initial OD (incubation = 0 h) from the final OD (incubation = 12 h).
12. OD around 0.01 or less was considered inhibition of growth of test organism.

2.13.1.3. Result

The minimum inhibitory concentration (MICs) of all 30 lead molecules against Gram negative, Gram positive and few MDR pathogenic bacteria is given in the Table.2.1. Many of these compounds were effective against a broad range of microbes including some multidrug resistant (MDR) pathogens.

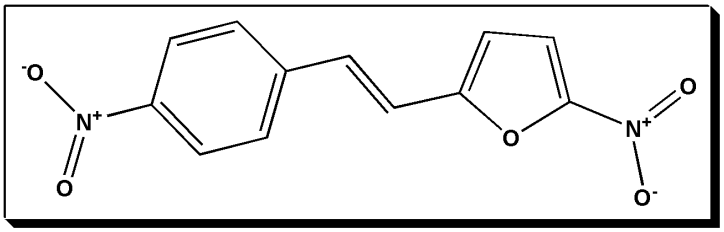
Table 2.1 MIC values of 30 lead molecules against Gram positive and Gram negative bacterial strains. Kanamycin sulphate was an antibiotic control to ensure that the assay is working well. Multiple drug resistant *E. coli* O157 and *Vibrio fluvialis* (BD-146) were kind gift of Dr. Amit Ghosh. All the other strains used in the study were purchased from ATCC. The MIC was determined by two fold microbroth dilution method in 96-well plate according to CLSI (Clinical and Laboratory Standards Institute) guidelines.

Sr. No.	IITR Code	Gram negative MIC in µg/ml						Gram positive MIC in µg/ml
		E. coli ATCC 25922	*Enterobacter sakazaki*	*Shigella flexneri*	*E. coli O 157*	*Acinetobacter baumannii*	*Vibrio fluvialis*	*Bacillus cereus*
1	IITR 00205	13.50	27.00	13.50	13.50	54.00	13.5	6.75
2	IITR 00210	4.63	4.63	4.63	4.63	4.63	2.3	9.25
3	IITR 00693	33.00	8.25	4.13	16.50	16.50	33.0	8.25
4	IITR 00803	10.88	10.88	10.88	43.50	5.44	5.4	21.75
5	IITR 01245	53.25	106.50	26.63	106.50	13.31	26.6	26.63
6	IITR 01318	14.19	56.75	56.75	56.75	14.19	14.2	28.38
7	IITR 01324	3.58	28.63	3.58	14.31	57.25	28.6	14.31
8	IITR 02061	72.50	72.50	72.50	72.50	72.50	72.5	72.50
9	IITR 06033	61.00	15.25	1.90	7.63	15.25	15.3	7.63
10	IITR 06144	0.51	0.51	0.13	0.25	1.02	0.1	2.03
11	IITR 06146	6.16	12.31	3.08	6.16	49.25	1.5	3.08
12	IITR 06567	8.25	16.50	16.50	16.50	66.00	33.0	66.00
13	IITR 07064	1.62	1.63	26.00	0.81	52.00	26.0	52.00
14	IITR 07273	64.50	64.50	8.06	64.50	32.25	16.1	2.02
15	IITR 07347	18.75	18.75	9.38	37.50	9.38	9.4	18.75
16	IITR 07480	18.50	9.25	18.50	18.50	2.31	18.5	37.00
17	IITR 07711	15.19	3.80	3.80	7.59	7.59	7.6	30.38
18	IITR 07806	20.81	83.25	10.41	83.25	41.63	10.4	2.60
19	IITR 07865	30.75	7.69	61.50	1.92	61.50	15.4	30.75
20	IITR 07938	22.38	44.75	11.19	44.75	44.75	22.4	11.19
21	IITR 07940	27.25	27.25	1.70	27.25	13.63	3.4	1.70
22	IITR 07942	20.38	40.75	10.19	40.75	40.75	10.2	10.19
23	IITR 07952	32.25	64.50	32.25	64.50	64.50	4.0	8.06
24	IITR 08276	6.41	12.81	3.20	12.81	6.41	3.2	6.41
25	IITR 08345	15.38	30.75	7.69	30.75	15.38	3.8	30.75
26	IITR 08524	12.56	50.25	1.57	12.56	12.56	3.1	12.56
27	IITR 08540	62.00	62.00	1.94	62.00	31.00	7.8	15.50
28	IITR 08548	22.63	45.25	2.83	45.25	45.25	5.7	11.31
29	IITR 08564	13.63	54.50	6.81	54.50	13.63	1.7	6.81
30	IITR 08599	6.06	48.50	6.06	24.25	6.06	6.1	12.13
31	Kanamycin	4.00	1.50	1.50	3.00	0.75	1.5	1.50

2.14. Identification of IITR06144, a novel broad spectrum antibacterial small molecule

Amongst the 30 major leads identified after primary screening, IITR06144 ((E)-2-(4-nitrostyryl)-5-nitrofuran) was the most potent antibacterial molecule against *E. coli* (Fig. 20). The minimum inhibitory concentration of IITR06144 against *E. coli* ATCC25922 was 0.5 μg/ml. The nitro-aromatic molecule of 260 Da is a fusion of a nitro-benzene and a nitro-furan moiety linked by carbon-carbon double bond. The molecule can be synthesized by condensation of 5-nitro-2-furaldehyde and 4-nitrobenzaldehyde.

The minimum inhibitory concentrations (MICs) of both these molecules were determined against *E. coli* ATCC25922. It was observed that the MIC of furaldehyde was much lower than benzaldehyde molecule. The MICs of 5-nitro-2-furaldehyde and 4-nitrobenzaldehyde are 1.5μg/ml and 25 μg/ml. This finding suggests that the nitro furan region of the molecule is imparting the higher potency of the entire molecule. When these two moieties condense to form IITR06144, it becomes more potent. The MIC of IITR06144 against *E. coli* ATCC25922 was found to be 0.5 μg/ml which was lower than the MICs of both the individual aldehyde molecules (Fig. 21)



IITR06144

(*E*)-2-(4-nitrostyryl)-5-nitrofuran

Figure 2.20 Structure of IITR06144, a novel antibacterial small molecule.

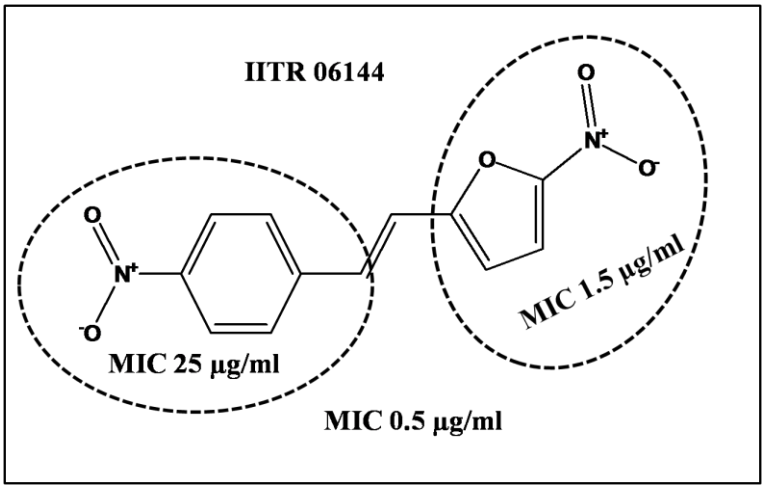


Figure 2.21 Analysis of two structural components of IITR06144.

2.15. Determining the antibacterial spectrum of IITR06144

Susceptibility of IITR06144 was tested against a broad range of bacterial pathogens which include Gram negative, Gram positive and multidrug resistant clinical isolates. The MIC of the molecule against various organisms was determined by two fold broth microdilution method in 96-well plate as described in previous section 2.13.1.

Table 2.2 MIC values of IITR06144 in comparison to a nitro aromatic drug (nitrofurantoin) and a standard antibiotic control (kanamycin) against a broad range of bacteria including Gram negative, Gram positive and multidrug resistant bacteria. The MIC was determined by two fold microbroth dilution method in 96-well plate according to CLSI (Clinical and Laboratory Standards Institute) guidelines.

Sr.No	Name of Microorganism	MIC in µg/ml		
		IITR06144	**Nitrofurantoin**	**Kanamycin**
Gram negative				
1	*Escherichia coli* ATCC 25922	0.5	8	4
2	*Escherichia coli* BW25113	0.5	8	2
3	*Escherichia coli* MG1655	0.5	8	2
4	*Cronobacter sakazakii*	0.5	8	2
5	*Acinetobacter baumannii ATCC17978*	0.25	> 64	0.5
6	*Acinetobacter baumannii SDF*	2	64	0.25
Gram positive				
13	*Staphylococcus aureus*	2	16	2
14	*Bacillus subtilis*	2	16	2
15	*Bacillus cereus* ATCC 11778	0.5	16	4
16	*Listeria monocytogenes*	0.5	8	>32
17	*Enterococcus faecium*	4	32	>32
18	*Clostridium perfringens*	0.5	8	>32
Gram negative (MDR)				
19	*Vibrio fluvialis (*L-15318)	0.13	2	16
21	*Vibrio fluvialis* (BD146)	0.13	2	64
20	*Escherichia coli 0157*	0.25	8	4
22	*Acinetobacter baumannii AYE*	0.6	32	>64

2.16. Determining the toxicity of IITR06144

To be a good antibacterial, it is desirable that the molecule should not be toxic to the animal cells. The toxicity of IITR06144 was determined by hemolysis assay and MTT assay.

2.16.1. Hemolysis Assay

The hemolysis assay measures the toxicity in terms of lysis potential of a molecule to human red blood cells. Higher the lysis potential of a molecule, lesser the potential of a good antibacterial. The toxicity of IITR06144 was determined by hemolysis assay.

2.16.1.1. Materials

0.9 % NaCl solution, Dulbecco's phosphate buffer saline (D-PBS), Triton-X100, DMSO, IITR06144, nitrofurantoin, furazolidin.

2.16.1.2. Method

1. Fresh blood (2.5 ml) was collected from a healthy individual in a collection tube containing anticoagulant.
2. Blood sample was immediately centrifuged at 1500X g for 5 min. This process separated the red blood cells from the serum.
3. The serum portion from the top was removed carefully by using a pipette and the red blood cells were dispensed in 0.9 % NaCl saline solution approximately 9 times of the volume of the red blood cells.
4. The suspension was again centrifuged at 1500Xg for 5 min and the supernatant was removed carefully by using a pipette. The process was repeated twice.
5. After each washing, cells were pelleted by centrifugation at 1500X*g* for 5 min and the supernatant was discarded.
6. The final pellet was diluted 1:9 (v/v) in sterile 0.9 % NaCl saline solution than 1:24 (v/v) in sterile Dulbecco's phosphate buffer saline (D-PBS), pH 7.0.
7. Red cell suspensions (1 ml of final volume) were incubated at 37°C for 6 h with different concentration (1X, 5X, 10X and 20X of MIC values against *E. coli* ATCC25922) of IITR06144, nitrofurantoin and furazolidine.
8. For each compound, red cell suspension with same volume of solvent (DMSO) were also incubated at same condition for 6 h. (Solvent control).
9. In order to avoid any possible differences due to a diverse manipulation, also samples without any treatment were also incubated for same period at 37°C. Red cell suspensions without any treatment was taken as negative control (minimum hemolysis).
10. Another suspension was incubated with Triton-X100 (1% v/v) which was used as a positive control. Hemolytic potential of test compounds were measured in comparison to the positive control. Hemolysis caused by Triton-X 100 was considered as 100 %.

11. During the incubation the samples were occasionally (maximum once per h) resuspended by inversion.
12. For each concentration and control, the experiments were set in triplicate.
13. Hemolysis was measured in terms of measuring the absorbance at 540 nm.

Percentage hemolysis = (OD_{540} Test - OD_{540} Solvent control) / (OD_{540} Positive control - OD_{540} Solvent control) x 100

2.16.1.3. Result

It was observed that the percentage hemolysis by IITR06144 was quite similar with the two other nitro-aromatic antibacterial drugs available in the market. Hemolytic potential of all the molecules in comparison to Triton X 100 (1% v/v) were below 25 %. The observation is given in the following table (Table.2.3).

Table 2.3 Percentage hemolysis of IITR06144 and two other nitro-aromatic drugs in comparison to Triton X 100 1% (v/v). Hemolysis by Triton X 100 was considered as 100%.

Drug Concentration	Percentage haemolysis ± Standard deviation		
	IITR06144	Nitrofurantoin	Furazolidine
1X MIC	9 ± 1	10 ± 1.5	7 ± 1
5X MIC	10 ±1	14 ± 2	6 ± 1
10X MIC	14 ± 1.6	20 ± 2.1	11 ± 2
20X MIC	18 ± 1.6	21 ± 1.5	15 ± 2

2.16.2. MTT Assay

The MTT assay was carried out against the stem cells C3H10T1/2. This assay measures the toxicity of an antibacterial molecule in terms of cell viability after a brief exposure of cell line to the molecule at various concentrations. Higher the percentage viability after a brief exposure, greater is the utility of a good antibacterial.

2.16.2.1. Materials

C3H10T1/2 stem cells, IITR06144 (Maybridge, UK), nitrofurantoin (Sigma Aldrich), furazolidone (Sigma Aldrich) Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific), MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide](Thermo Fisher Scientific), DMSO (Merck, Germany), 96-well tissue culture plate flat bottom, CO_2 and cell culture facility.

2.16.2.2. Method

1. Approximately 5×10^3 C3H10T1/2 cells in 200 µl of media were seeded in a 96-well microtiter plate and incubated overnight.
2. Cells were then treated with 1 µl of different concentrations of antibacterials (IITR06144, nitrofurantoin and furazolidone) and incubated at 37°C inside a CO_2 incubator for 24 h.
3. Control cells were treated with 0.5 % DMSO (vehicle control) and incubated at same condition for 24 h.
4. After 24 h, 20 µl MTT (5 mg/ml) were added to the culture and incubated for 4 h at 37°C.
5. MTT-containing medium was then removed very carefully by a pipette holding the plate in a tilted way. The purpose was not to disturb the formazone layer at the bottom.
6. 200 µl DMSO was added to dissolve the formazone crystal by gentle agitation.
7. Optical density (OD) was measured at 570 nm in a plate reader (M2e, Molecular Devices).
8. Viability was measures in terms of absorbance values. Percentage viability was calculated in comparison to the solvent control.
9. The percentage viability was calculated by using the following formula.

Percentage cell viability = [(Mean OD of treated cell×100)/mean OD of vehicle treated cells] X 100

2.16.2.3. Result

A dose dependent decrease in cell viability was observed for IITR06144 and other two nitroaromatic drugs available in the market. However, at MIC concentration, the molecules exhibit a very low toxicity.

Table 2.4 Percentage viability of IITR06144 in comparison to two other nitro-aromatic drugs nitrofurantoin and furazolidine. Viability of cells treated with vehicle (DMSO 0.05%) was considered as 100%.

Drug Concentration	Percentage cell viability ± Standard deviation		
	IITR06144	**Nitrofurantoin**	**Furazolidine**
1X MIC	95 ± 4	81 ± 9	87 ± 7
5X MIC	55 ± 5	48 ± 4	61 ± 5
10X MIC	40 ± 4	40 ± 4	51 ± 6
20X MIC	25 ± 5	40 ± 6	15 ± 3

2.17. Determine the mode of action of IITR06144

In order to ascertain any changes in cell morphology, microscopic studies of *E. coli* cells after a brief exposure to IITR06144 were carried out using light microscopy and bright field microscopy, followed by scanning electron microscopy. The morphological change in bacterial cells with and without the treatment of the drug was monitored by microscopic observation.

2.17.1. Microscopic study

2.17.1.1. Materials

Glass slides, small glass slides (for SEM), cover slips, microcentrifuge tubes, 0.9% NaCl solution, glutaraldehyde (4%), ethanol, Gram staining solutions (Himedia).

2.17.1.2. Methods

2.17.1.2.1. Preparation of cells

1. A single well isolated colony of *E. coli* ATCC25922 was inoculated from fresh LB-Agar plate into 5ml sterile LB broth in a culture tube and incubated at 37°C with 200 rpm for 6 h.
2. Cells were then sub cultured (0.1%) in two other fresh tubes with 5 ml of media and incubated at same condition.
3. OD_{600} of the cells were allowed to come around 0.1.
4. One of the tubes was treated with 0.25 µg/ml of IITR06144.
5. Both the tubes were incubated for two h and smear was prepared immediately on grease free clean slide for microscopic observation.

2.17.1.2.2. Sample preparation for brightfield and fluorescence microscopy

1. Prepared cells were pellet down by centrifugation at 6000rpm/10 min at 4°C.
2. Supernatant was discarded and cells were resuspended in 1ml of 0.9 % saline.
3. Cells were again centrifuged and resuspended in 1 ml of 0.9% saline.
4. A drop of the suspension was put on the clear grease free glass slide and a thin smear was prepared.
5. Sample was then allowed to air dry for few min.
6. Dried smear was than heat fixed by heating gently from a distance to a spirit lamp for few s. Over heating was avoided.
7. For fluorescence and phase contrast imaging no staining is required. Smear was ready for the microscopy after putting a cover slip on it.
8. For brightfield microscopy smear was stained as per the method of Gram staining.

2.17.1.2.3. Sample preparation for SEM (Scanning Electron Microscopy) imaging

1. The prepared cells were washed once with 0.9 % NaCl and resuspended in 0.9% NaCl solution.
2. To fix the cell, equal volume (100 µl) of 4% gultraldehyde solution prepared in 0.9% saline was added to make the final concentration as 2% into a microcentrifuge tube.
3. The samples were incubated at 4°C for 30-45 min.
4. A very small droplet of the sample was put on very small grease free clean glass slide and allowed to air dry to make very a small circular smear. If required, the droplet was spread gently on glass surface with the sharp end of a microtip of 10 µl capacity.
5. Cells were allowed to dry up by incubating at 40°C for 10 -15 min.
6. After the smear dried up, samples were dehydrated by placing the slides in 25, 50, 70, 80, 90, 100% ethanol for 5 min each.
7. The samples were allowed to be dried by incubating at 37°C for 10-15 min.
8. Gold coating on samples were performed using Q150R Rotary-Pumped Sputter Coater and samples were visualized using Zeiss ULTRA-PLUS SEM microscope.

2.17.1.2.4. Gram Staining procedure

1. Heat fixed slide was placed on a staining tray and the smear was covered with crystal violet for 1 min.
2. The liquid stain was washed off with water (not more than 2-3 s).
3. Smear was then covered with iodine solution for 1 min and then washed off with water.
4. The slide was blot dried to remove excess water.
5. Decolorization of smear with 95% ethanol was carried out by dripping ethanol on smear with slide held at right angle until the dye stops coming out.
6. Then the slide was flooded with water and blot dried.
7. The smear was then flooded with saffranine for 1 min.
8. Slide was rinsed briefly with water to remove excess stain.
9. The slide was then left for air drying and then microscopically examined under 100X oil immersion objective lens.

2.17.1.3. Results

Change in morphology of *E .coli* cells after exposure to IITR06144 was monitored by thorough microscopic studies. It was observed the brief exposure of IITR061444 at sub MIC concentration causes cellular elongation of *E. coli* cells (Fig.2.22-2.23). It was also found that

there was constrain in separation of two daughter cells during cell division (Fig.2.24). The SEM images confirmed that the cells fail to separate from each other after treatment with IITR06144 leads to elongation. Therefore it was concluded that IITR06144 restricts bacterial cell division leading to cellular elongation (Fig.2.25).

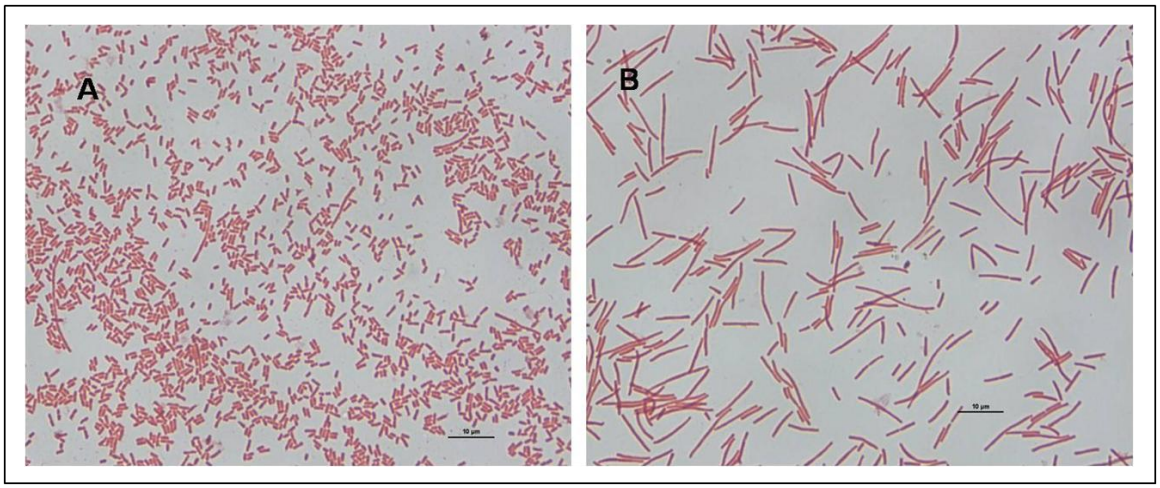


Figure 2.22 Bright field (100X objective) microscopy images of *E. coli* cells after Gram staining. (**A**) cells without treatment of any drug, (**B)** cells treated with sub MIC concentration of IITR06144.

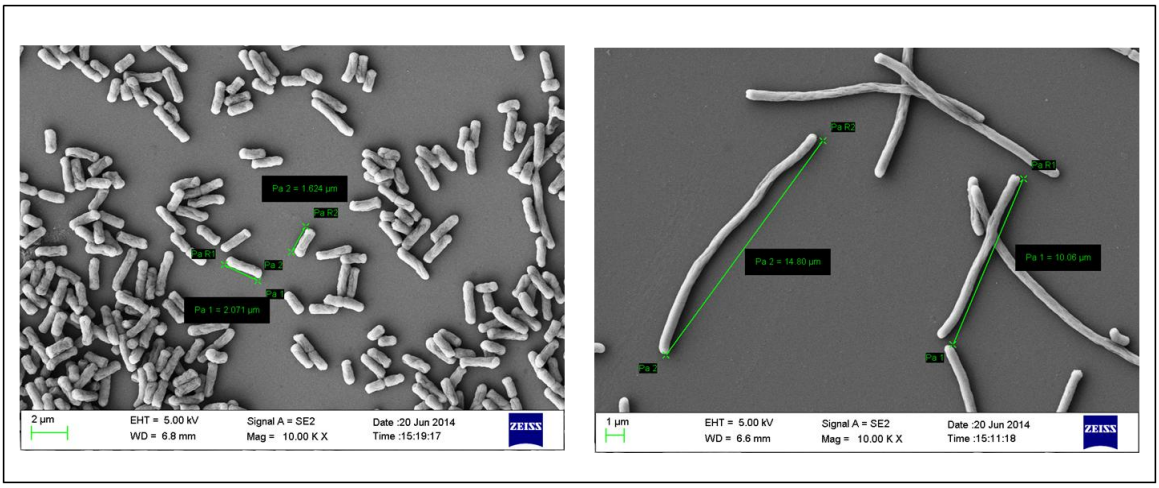


Figure 2.23 Scanning electron microscopy images of *E. coli* cells. (**A)** cells without treatment of any drug, (**B)** cells treated with sub MIC concentration of IITR06144.

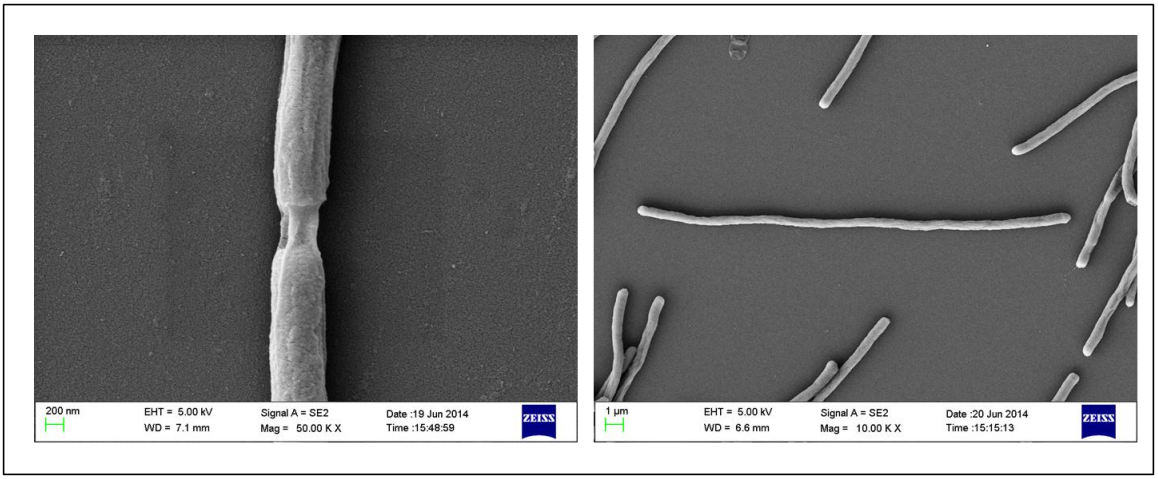


Figure 2.24 Scanning electron microscope (SEM) image of *E. coli* cells treated with sub MIC concentration of IITR06144. (**A)** Two daughter cells not getting separated properly, (**B)** Elongated cells after treatment with IITR06144.

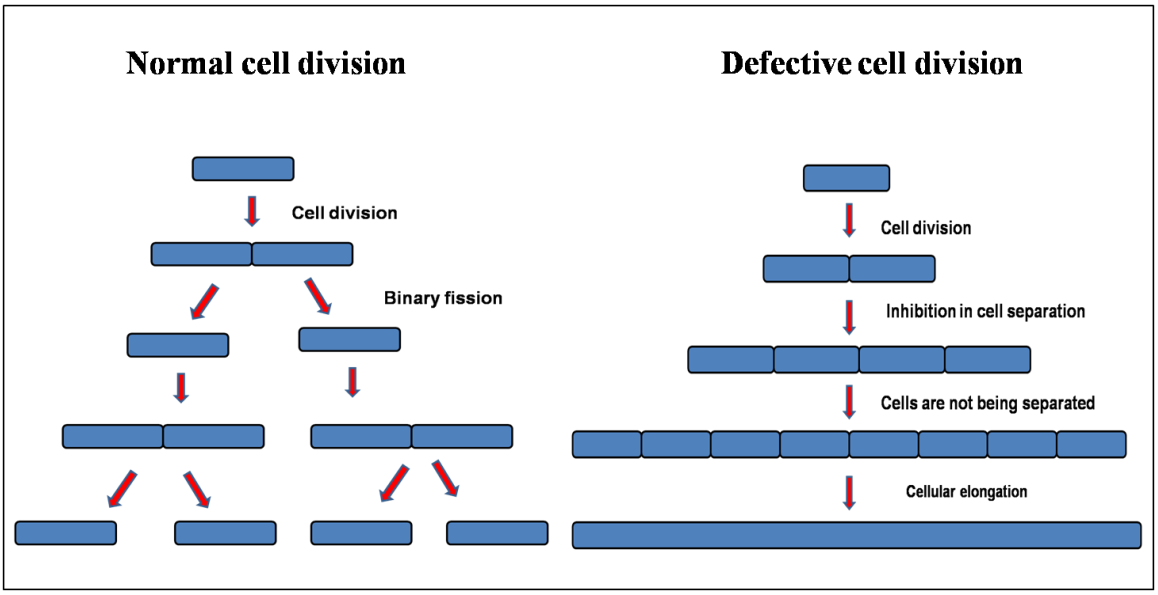


Figure 2.25 Schematic representation of normal and defective cell division.

2.17.2. Studies on *E. coli* cells overexpressing FtsZ-GFP

FtsZ and its associated protein family are the key players in bacterial cell division. Polymerization of FtsZ causes the formation of septum or division ring during the cell division. Inhibition in FtsZ assembly formation may lead to the defective cell division and subsequent cellular elongation. Therefore, the effect of IITR06144 on the assembly of FtsZ was investigated in details. *E. coli* cells harboring a recombinant plasmid pCA24N was used in the study [70]. This is an inducible plasmid carrying f*tsZ-gfp*. Induction for the expression of FtsZ also causes an overexpression of GFP which can be monitored by fluorescence.

2.17.2.1. Materials

E. coli (pCA24N-*ftsZ-gfp*) cells, LB Broth, IPTG, chloramphenicol, culture tubes and fluorescence microscope.

2.17.2.2. Method

1. A single colony of *E. coli* (pCA24N-*ftsZ-gfp*) was picked from a fresh LB Agar plate with *chloramphenicol* (30 μg/ml) and inoculated into 5 ml of LB broth in a culture tube with same concentration of antibiotic.
2. The tube was incubated at 37°C with 250 rpm until the OD_{600} reaches at 0.5.
3. At this level, the culture was induced by 1 mM of IPTG (50 μl of 100 mM stock solution of IPTG) and incubated at same condition for 2 h.
4. The cells were then diluted to OD_{600} of 0.1 in two other fresh tubes containing 5 ml LB broth with chloramphenicol (30 μg/ml) and IPTG (0.5 mM).
5. In one tube the cells were treated with 0.25 μg/ml of IITR06144 and the other tube was kept as a control without any treatment.
6. The tubes were incubated at 37°C, 250 rpm for 4 h.
7. The culture tubes were removed and smear was prepared on clean glass slide immediately as described in previous section 2.17.1.2.2
8. The slides were observed under an inverted fluorescence microscope (Nikon, Ti Eclipse) with 100X oil emmersion objective.

2.17.2.3. Result

When the cells were induced with 1 mM IPTG simultaneous overexpression of FtsZ and GFP occurred. As a result, fluorescence of GFP was visible at uniform distance at mid cell region (Fig.2.26A). It means, without any treatment with IITR06144 the FtsZ was localized uniformly along the dividing cells. But, in case of cells treated with IITR06144 GFP was observed to be delocalized and dissolute in entire cells (Fig.2.26B). For a single cell also, the changes was very prominent (Fig.2.26C and 2.26D). This observation suggests that IITR06144 interferes with the formation of FtsZ assembly in the mid-cell region and as a consequence *E. coli* cell division was restricted leading to elongation of cells.

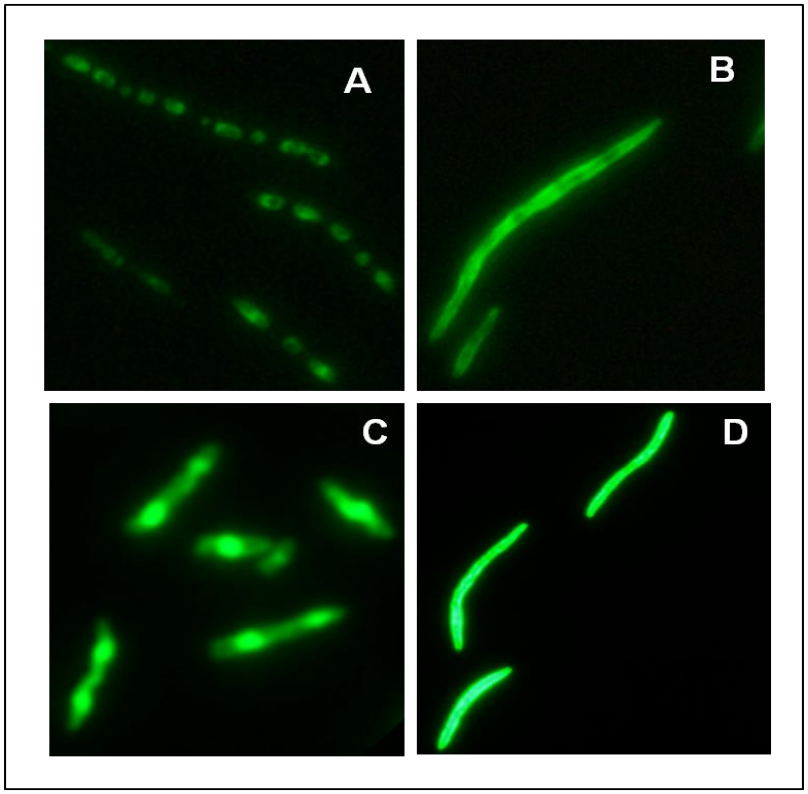


Figure 2.26 Fluorescence microscope image of *E. coli cells* harboring pCA24N-FtsZ-GFP (Aska library). **(A)** and **(C)**. Induced cells not treated with IITR06144 (control), **(B)** and **(D)**. Induced cells treated with sub MIC concentration of IITR06144. Delocalization of FtsZ-GFP was observed after treatment with IITR06144.

2.17.3. Antisense RNA mediated silencing for target identification

Antisense RNA of a particular gene binds to its sense mRNA and prevent its expression at further level. The phenomenon is known as antisense RNA mediated gene silencing. A system can be developed where antisense RNA mediated gene silencing can be achieved [71]. In this system a large fragment of a particular gene is cloned in antisense direction into an inducible plasmid and transformed into the bacterial cells. Induction causes the formation of antisense mRNA which bind to the sense mRNA (transcribed from the genome) of the gene and restricts its entry into the translation. Antisense expression of an essential gene causes impaired growth in bacteria. A drug with similar target will elevate the effect by acting in synergy. However, drug with dissimilar target will not influence the process [45] . This phenomenon can be utilized for identifying or confirming a drug target on genetic basis.

This approach was used in the present study to identify the target. Initial observations from sections 2.17.1.3 and 2.17.2.3 suggested that IITR06144 might have some interaction with FtsZ. Therefore, plasmid, pHN678 with antisense *ftsZ* and antisense *murA* in *E. coli* BW25113 was used in the study. *murA* is an essential gene and was taken as a control.

2.17.3.1. Materials

Plasmid pHN678, (kind gift from Prof. Liam Good), *E. coli* (pHN678-*ftsZ*), *E. coli* (pHN678-anti-*ftsZ*), anti-*ftsz and* anti-*murA* was cloned in the lab by others [45], IPTG, *chloramphenicol*, LB Broth, LB Agar, Petri plates, 96-well plates and spectrophotometer.

2.17.3.2. Confirmation of impaired growth on induction of essential genes in *E. coli*

The first step of the experiment was to confirm that antisense system was functioning properly on induction. Therefore, a spot assay was performed on LB agar plate using *E. coli* (pHN678), *E. coli* (pHN678-*ftsZ*) and *E. coli* (pHN678-*murA*) with and without IPTG.

2.17.3.2.1. Method

1. A single colony of *E. coli* (pHN678), *E. coli* (pHN678-*ftsZ*) and *E. coli* (pHN678-*murA*) were picked from a different LB-agar plate with chloramphenicol (30 μg/ml) and inoculated into three individual culture tube containing 5 ml of sterile LB broth with equal concentration of antibiotic.
2. The cultures were incubated at 37°C for 12 h shaking (200 rpm).
3. The saturated culture was diluted 100 fold in sterile LB broth with chloramphenicol (30 μg/ml).
4. 5 μl of the culture was spotted (in triplicate) on to LB agar pate with and without IPTG (1.5 mM). Both the plates contained chloramphenicol at 30 μg/ml concentration.
5. The spots were allowed to dry up for few min inside the biosafety cabinet.
6. Both the plates were incubated for 12 h and observed for growth of cells.

2.17.3.2.2. Result

Impaired growth pattern was observed for both the antisense clones, *E. coli* (pHN678-*ftsZ*) and *E. coli* (pHN678-*murA*) in the plate containing 1.5 mM IPTG (Fig.2.27B). No defective growth was observed for the cells harboring only pHN678 plasmid. All the cells displayed good growth in controlled conditions where no IPTG was added (Fig.2.27A). It suggested that the antisense system was functioning well.

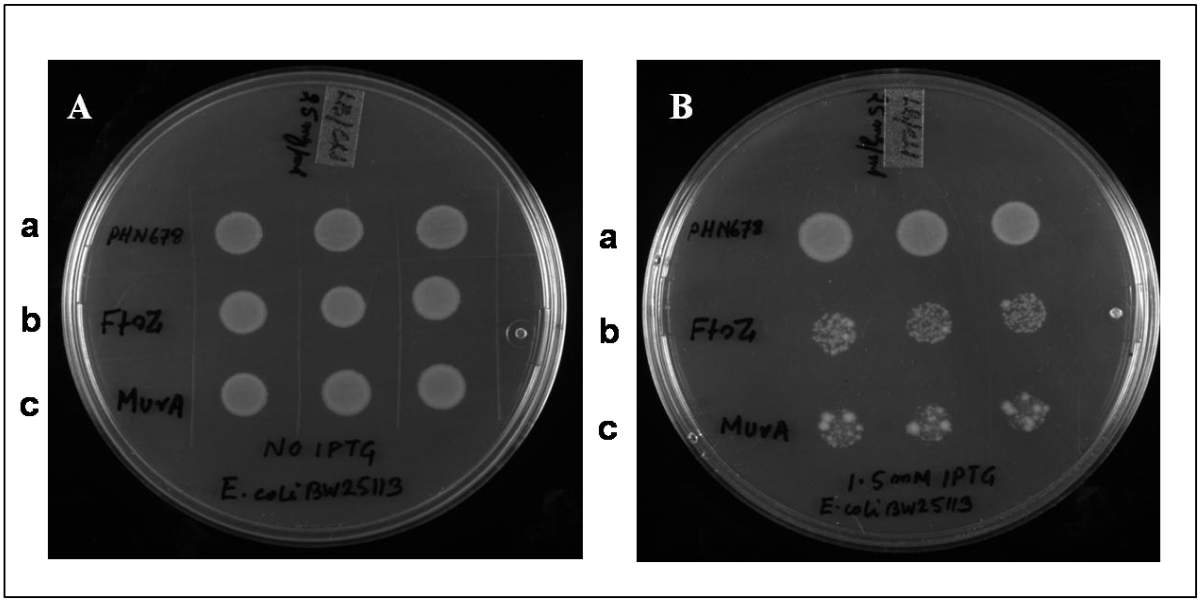


Figure 2.27 Spot assay of *E. coli* BW25113 cells harboring different plasmids (a) *E. coli* (pHN678) (b) *E. coli* (pHN678-*ftsZ*) and (c) *E. coli* (pHN678-*murA*). **(A)** LB agar plate without IPTG (uninduced), **(B)**. LB agar plate with IPTG (induced). Three different spots were made for each strain in both the plates.

2.17.3.3. Determination of the growth profile of both the antisense cells E. coli (pHN678-ftsZ) and E. coli (pHN678-murA) at different induction level in combination with IITR06144

2.17.3.3.1. Method

1. Different panels in 96-well plates were filled with 100 µl of LB broth with different concentrations (70 µM, 150 µM and 300 µl) of IPTG and IITR06144 (0.4 µg/ml, 0.2 µg/ml and 0.1µg/ml) in triplicates.
2. Three wells for each concentration of IPTG were kept without any small molecule.
3. Another panel in 96-well plate was filled with 100 µl of LB broth without any IPTG. The concentration of IITR06144 was 0.4 µg/ml, 0.2 µg/ml and 0.1µg/ml.
4. Three wells without the IPTG were kept without any small molecules.
5. The concentration of both IPTG and antibiotic was kept double of its desired concentration because it was diluted to half of its concentration when 100 µl of inoculum was added.
6. LB broth with chloramphenicol (30 µg/ml) was used throughout the experiment.
7. Overnight grown saturated culture (as described in section 2.17.1.2.2) was diluted 10^3 fold in LB chloramphenicol media.
8. 100 µl of this diluted culture was dispensed in all the wells.

9. The plates were incubated at 37°C inside a humidity controlled incubator at shaking condition (100 rpm).
10. The plates were read at 600 nm using a plate reader at 1 h interval for 12 h.
11. Average changes in OD at 600 nm at different growth conditions were calculated and plotted against the time interval.

2.17.3.3.2. Result

Treatment with IITR06144 resulted in less growth of *E. coli* (pHN678-*ftsZ*) as compared to *E. coli* (pHN678-*murA*) cells observed. With increasing level of induction and increasing concentration of IITR06144 the growth profile of anti-*ftsZ* was affected significantly (Fig.2.28). However, no such changes were observed for anti-*murA* which was also an essential gene. The silencing of *ftsZ* was found to assist IITR06144 in its antibacterial activity even at lower concentration.

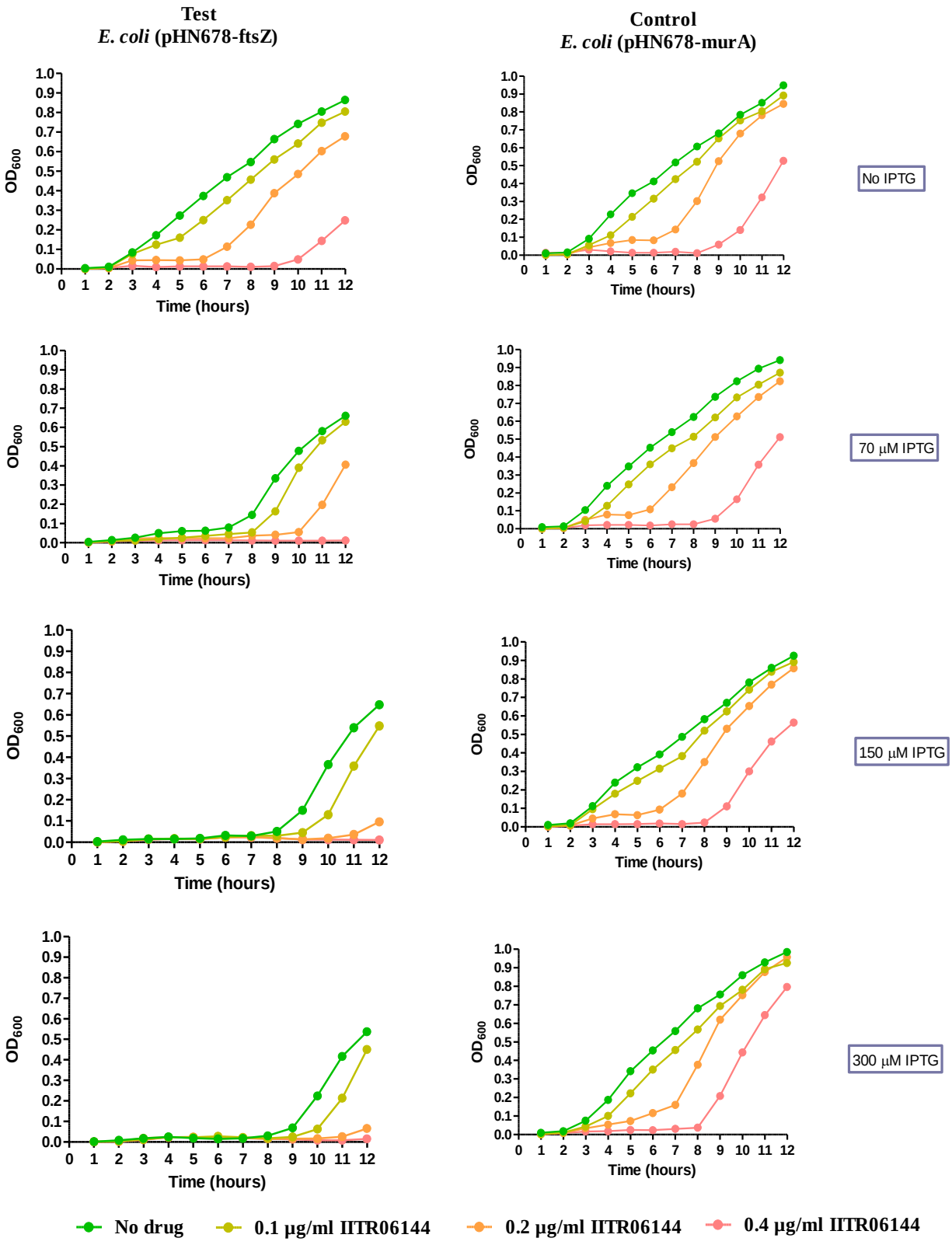


Figure 2.28: The growth profile of *E. coli* (pHN678-ftsZ) and *E. coli* (pHN678-murA) at different induction level in combination with different concentration of IITR06144.

2.17.4. Studies with the knockout mutants (Keio collections) of *E. coli*

Microscopic and genetic evidences suggested that IITR06144 interacts with bacterial cell division protein FtsZ. There are several internal players which also interact with FtsZ and regulate its polymerization during the septum formation. SlmA and MinC are the members of this category which negatively regulate FtsZ polymerization. The inhibition of FtsZ by IITR06144 may be regulated via any of these two members. Therefore, the susceptibility of these knockout mutants of *E. coli* was tested against IITR06144. IITR06144 is a nitroaromatic compound which like other nitro aromatic compounds may be reduced inside the cells by major reductases, NfsA and NfsB. Therefore, deletion mutant of these reductases were also studied.

2.17.4.1. Method

The Minimum inhibitory concentration of IITR06144 was determined against these knockout mutants in comparison to the wild type *E. coli* BW25113 by two fold broth microdilution method in 96-well plate with liquid media as described in section 2.13.1.

2.17.4.2. Result

MICs of IITR06144 against the knockout mutants (Keio collections) of *E. coli* are given in Fig.2.29. The MIC of IITR06144 was found higher for ΔnfsA mutant in comparison to the wild type *E. coli*. The finding suggests that NfsA is required for better potency of IITR06144. However, NfsB does not play any role for functioning of IITR06144 as an antibacterial. The MIC of IITR06144 against Δ*slmA* and Δ*minC* was two folds lower than the wild type stain. It suggests that SlmA and MinC have a role in host immunity against the IITR06144. It is already known that SlmA and MinC function as a cellular check point during the cell division. Again, it confirms the fact that IITR06144 must be involved in restriction of cell division.

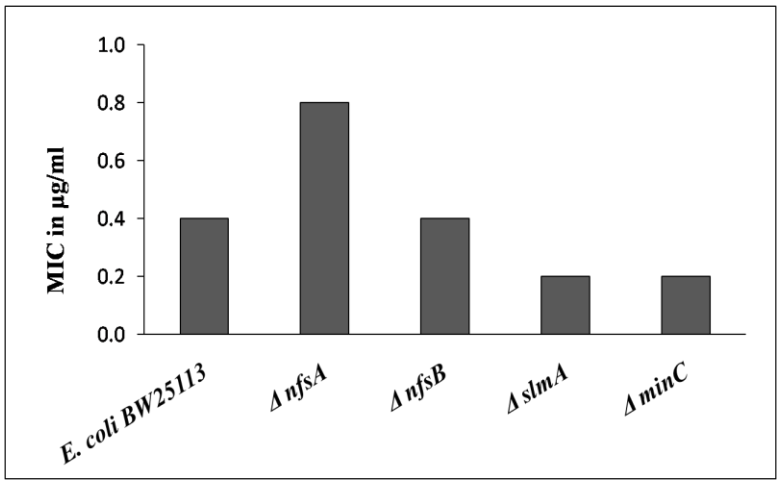


Figure 2.29 MIC of IITR06144 against the knockout mutants of *E. coli* in comparison to the wild type BW25113 cells.

2.17.5. Determining the interaction of IITR06144 and NfsA

Elevation in MIC of IITR06144 against the Δ*nfsA* suggested that the gene products assists the function the molecules as an antibacterial. To reconfirm the role of NfsA, MIC of IITR06144 was determined against the NfsA overexpressor (Aska library).

2.17.5.1. Method

The MIC was determined by broth microdilution method in 96-well plate as described in previous section 2.13.1.The liquid media used in assay was supplemented with 0.1 mM IPTG.

2.17.5.2. Result

Over expressing mutant of NfsA exhibited hyper susceptibility to IITR06144. It re-established the fact that NfsA enhance the activity of IITR06144.

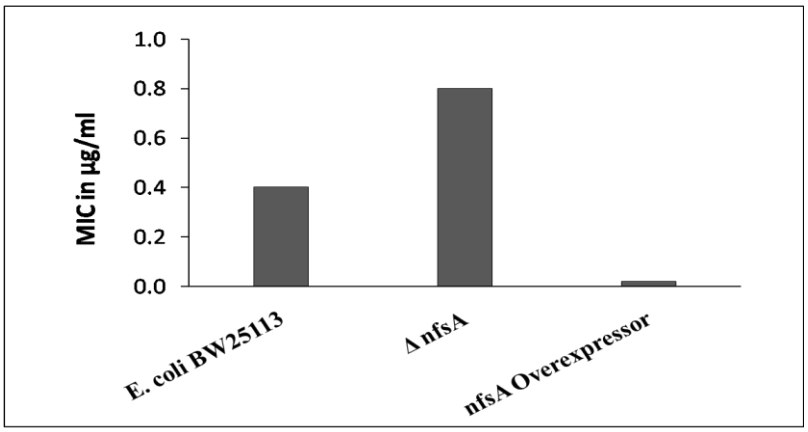


Figure 2.30 MIC of IITR06144 against the deletion and overexpression mutant of *E. coli.*

This study demonstrated a very comprehensive strategy of screening a small molecule library for identifying novel antibacterial molecule against Gram negative bacteria. Screening led to the identification of a broad spectrum novel antibacterial molecule, IITR06144. Microscopy data suggested that the molecule interferes with bacterial cell division, in particularly with the formation of FtsZ assembly during cell division leading to cellular elongation. Study based on the antisense mRNA mediated gene silencing strongly suggest that FtsZ seems to be the target of IITR06144.

Permission letter from Prof. Rothfield for Figure 2.2 (Page 11)

From : bhattacharyya.tapas@gmail.com (Tapas Bhattacharyya)
To
Lawrence I. Rothfield

Sir,
My name is Tapas Bhattacharyya. I am a PhD Student at Department of Biotechnology, Indian Institute of Technology Roorkee (IIT Roorkee), India. I am working under the supervision of Dr.Ranjana Pathania, Associate Professor, Department of Biotechnology, IIT Roorkee.
I am going to submit my thesis very soon. The title of my thesis is "Chemical Genetic Approaches To Combat Multiple Drug Resistance In Gram-Negative Bacteria". In my thesis, I want to use one figure (Fig.1) from "Bacterial Cell Division: The Cycle of the Ring" (http://www.sciencedirect.com/science/article/pii/S0092867400818991)
to explain the formation of FtsZ assembly during cell division.

I am seeking your permission for using this figure from your research article. If you please allow me to use this image in my thesis, I shall be grateful to you.

Thanking you.

Sincerely
Tapas Bhattacharyya

Reply from: lroth@uchc.edu (Lawrence I. Rothfield)

Dear Tapas,
You have my permission to use the figure.
Good luck!,
Larry Rothfield

Nh

Sent from my iPad

Lawrence Rothfield
Dept. of Molecular Biology and Biophysics
University of Connecticut Helth Center
363 Farmington Ave.
Farmington, CT 06032
Telephone- 860-922-7564

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF NOVEL EFFLUX PUMP INHIBITORS (EPIs)

CHAPTER 3

IDENTIFICATION AND CHARECTERIZATION OF NOVEL EFFULUX PUMP INHIBITORS (EPIs)

3.1. Efflux pumps

Efflux pumps in bacterial membranes are trans-membrane protein or protein assemblies that extrude toxic chemical substances including antibiotics from within the cell to outer environment. An efflux pump may have a specific substrate or it may transport structurally dissimilar multiple classes of chemicals or antibiotics [72].One of the major causes of multidrug resistance in bacteria is the presence of efflux pumps or transporter proteins in their membrane [73-75]. Multi subunit efflux systems in Gram negative bacteria, particularly in *Pseudomonas aeruginosa*, *Acinetobacter spp. Stenotrophomonas maltophilia*, and the *Enterobacteriaceae* have been recognized as the major determinants of intrinsic as well as acquired multidrug resistance in these organism [76]. Efflux pumps are primarily categorized into two major groups, based on their driving force for transporting the substrate across the membrane. ATP driven transporters like ABC (ATP-binding cassette) transporters are primary transporters which use the energy of ATP hydrolysis. The others are secondary transporters which utilize the electrochemical gradient of various ions across the membrane as a source of energy. Important secondary transporters includes RND (Resistance Nodulation Division), MFS (Major facilitator superfamily), SMR (Small Multidrug Resistance) and MATE (Multidrug And Toxic Compound Extrusion). The secondary pumps are mostly associated with multiple drug resistance in bacteria.

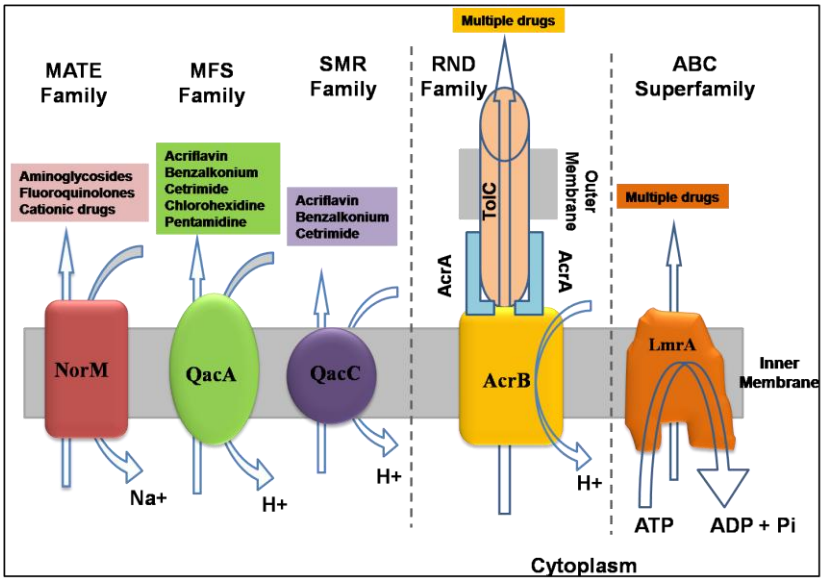


Figure 3.1 Clinically relevant efflux pumps in bacterial pathogens (According to Piddock *et al*., 2006).

3.1.1. *ABC Transporters*

ABC (ATP-binding cassette) transporters are multi subunit primary transporters present in almost every kind of cells in all the kingdom of life. The multi subunit system consist of two trans-membrane domain (TMDs) embedded in the membrane bilayer and two nucleotide binding domains (NBDs) located in the cytoplasm. These transporters are driven by energy of ATP hydrolysis. Binding of ATPs at the cytoplasmic domain causes the conformational change in the trans-membrane which leads to the transportation of a specific substrates [77]. ABC transporters function both as exporter and importer for translocation of molecules or nutrients to energetically unfavorable direction. One ABC transport system in *E. coli* is responsible for preventing the accumulation of phospholipid on the outer surface of the outer membrane [78].

3.1.2. *RND Transporters*

RND (Resistance Nodulation Division) is the most prevalent transmembrane efflux system found in Gram negative bacteria. This transport system consists of three components: an inner membrane domain, a periplasmic domain and an outer membrane domain. Many RND family pumps have been discovered and characterized in various gram negative pathogenic bacteria. The most studied transporter system belonging to RND family include AcrAB-TolC system of *Escherichia coli*, MexAB-OprM system of *Pseudomonas aeruginosa* and AdeABC, AdeIJK system of *Acinetobacter baumannii*. The RND family of pumps have a wide range of substrate specificity and are responsible for effluxing clinically significant antibiotics like aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones and β-lactams [79,80].

3.1.3. *MFS Transporters*

MFS (Major facilitator superfamily), also known as Uniporter-Symporter-Antiporter family is the most prevalent class of secondary transporter present in most of the living cells. Initially it was known for its role for uptake of sugars into the cells. Later it was discovered that it is also involved in the transportation of drugs and metabolites of Krebs cycle [81]. However, at least 7 different members of this category have been identified and characterized which are involved in multidrug resistance [82]. NorA of *Staphylococcus aureus* and PmrA of *Streptococcus pneumonia* are the most studied members of this category [15].

3.1.4. *SMR Transporters*

SMR (Small Multidrug Resistance) is another category of well characterized efflux pumps found in many bacteria. Most studied SMR family pumps are EmrE of *Pseudomonas aeruginosa*, EbrAB of *Bacillus subtilis*, SsmE of *Serratia marcescens* and AbeS of *Acinetobacter baumannii* [83]. On an average of 110 amino acid long SMR family transporters are generally located in inner membrane of gram negative bacteria and consist of four transmembrane helices. SMR family transporters are generally responsible for effluxing lipophilic drugs [84].

3.1.5. *MATE Transporters*

MATE (Multidrug And Toxic Compound Extrusion) is the most recently identified category of multidrug efflux pump among the other secondary transporters which use electrochemical gradient of cations across the membrane for transporting drugs [85]. This pump has diverse substrate specificity and expels several structurally dissimilar substrates including several clinically useful antibiotics from the bacterial cells [86,87]. Fluoroquinolones are the most common substrates for all the MATE family pumps identified so far. [85]. The important multidrug efflux pumps belonging to MATE family include NorM, BexA, VmrA, CdeA, YdhE, HmrM , PmpM, DinF and AbeM which have been identified in Gram negative bacteria [85,88,89]. *NorM* was the first reported Na^+ driven Na^+/drug antiporter identified in *Vibrio parahaemolyticus* [89] . A homolog of *norM* gene from *Vibrio parahaemolyticus* and *ydhE* from *E. coli* was latter identified in *Neisseria gonorrhoeae* [87] . VmrA is another Na^+ coupled multi drug transporter identified in *Vibrio parahaemolyticus* [88].Most recently, ketM, a multi drug efflux pump of MATE family, has been identified and characterized in *Klebsiella pneumonia* [90]. A novel MATE family efflux pump, mepA was identified in *Staphylococcus aureus* which cause decreased susceptibility of tigecycline [91]. In *Acinetobacter baumannii*, AbeM is the only MATE family pump identified and characterized so far.

3.2. Efflux pumps in *Acinetobacter baumannii*

Acinetobacter baumannii is one of the most troublesome, opportunistic, Gram negative bacillus associated with multiple nosocomial infections in humans [92-94]. Till 1970, the organism was susceptible to most of the antibiotics, but now it has become the leading cause of hospital acquired infection worldwide by acquiring resistance to most of the antibiotics [7]. There are many factors which contribute to drug resistance in *A. baumannii*. However, Multiple drug resistance in the organism is often associated with various efflux pumps which have been

identified in the organism [79,94,95]. A number of efflux pumps belonging to almost every classes of transporters have been identified in *A. baumannii* [15, 79,96]. AdeIJK, a multi subunit transporter belong to RND family was discovered in a clinical isolate of *A. baumannii* [80]. *adeABC and adeDE* gene clusters expressing multidrug transporters were found to function in association with *adeIJG* and distributed along the entire genome of *A. baumannii* [97]. AbeS, a multidrug efflux pump of SMR family was identified in the genome of the organism [83]. Amva, a MFS family pump that extrudes a number of antibiotics and disinfectants and confers resistance to the organisms against them [98]. The prevalence of various multi drug efflux pumps in *A. baumannii* poses a serious threat to the healthcare. The solution of the problem exists in the discovery of effective efflux pump inhibitors which can be prescribed along with the antibiotics to make them effective once again [18,73,99].

3.3. AbeM, a multidrug efflux pump in *Acinetobacter baumannii*

AbeM, a proton driven multidrug efflux pumps belonging to the MATE family of transporter was identified and characterized in *Acinetobacter baumannii* [86]. MATE (Multidrug And Toxic Compound Extrusion) is the most recently identified multidrug efflux pump among the other secondary transporters which use electrochemical gradient of cations across the membrane for transporting drugs [85]. This pump has diverse substrate specificity and expels several structurally dissimilar substrates including several clinically useful antibiotics from the cell [86,87]. Fluoroquinolones are the most common substrates for all the MATE family pumps identified so far in other bacteria also [85].

3.4. MATE family of transporters in other organisms

The other important multidrug efflux pumps belonging to MATE family include *NorM*, *BexA*, *VmrA*, *CdeA*, *YdhE*, *HmrM* , *PmpM* and *DinF* which have been identified in other Gram negative bacteria [85,88,89]. *NorM* was the first reported Na^+ driven Na^+/drug antiporter identified in *Vibrio parahaemolyticus* [89]. A homolog of *norM* gene from *Vibrio parahaemolyticus* and *ydhE* from *E. coli* was latter identified in *Neisseria gonorrhoeae* [87,88].VmrA is another Na^+ coupled multi drug transporter identified in *Vibrio parahaemolyticus* [88]. Most recently, ketM, a multi drug efflux pump of MATE family, has been identified and characterized in *Klebsiella pneumonia* [90]. In *Acinetobacter baumannii*, AbeM is the only MATE family pump which has been identified and characterized so far.

3.5. Efflux pumps as a drug targets

Efflux pumps can be excellent drug target because of their multiple substrate specificity. Inhibiting a particular efflux pump would revive the activity of number of clinically useful antibiotics. It may also reduce the mutant selection frequency against a particular antibiotic. Efflux pump inhibitors (EPIs) are also important for improving the pharmacodynamic characteristic like post antibiotic effect (PAE) of an antibiotic [100,101]. Efflux pump inhibitor against a multidrug efflux pump may also prevent the biofilm formation by the Gram negative pathogens [102]. As the discovery of new antibacterial drugs is in declining state, efflux pump inhibitors can play a significant role by rejuvenating the "old antibiotics" to replenish the void space.

3.6. Discovery of Efflux Pump Inhibitors (EPIs)

A number of efflux pump inhibitors has been identified and characterised against various transporters. Numerous strategies have been adopted for finding different families of transporters in different organisms. MBX2319**,** a pyranopyridine was identified and characterized as an efflux inhibitor of RND family pump in *Enterobacteriaceae* [103]. A novel molecule resembling the structure of piperine was discovered against the NorA efflux pump in *S. aureus* [104]. A set of efflux pump inhibitors were reported in *P. aeruginosa* which potentiate the activity of fluoroquinolones [105,106]. 1-(1-naphthylmethyl)-piperazine (NMP) was identified as an effective efflux pump inhibitor as it is capable of reversing MDR in *E. coli* over expressing the RND family of pumps and also reduces the MIC of several clinically useful antibiotics [107,108]. GG918, a synthetic efflux pump inhibitor potentiating the activity of fluoroquinolone against MDR *S. aureus* was discovered while screening to identify inhibitors of mammalian P-glycoprotein [109]. Verapamil was identified an excellent efflux pump inhibitor for *Mycobacterium tuberculosis* and profoundly reduces the MIC of bedaquiline against the strain [110]. Recently a novel serum associated efflux pump inhibitor was reported in *Acinetobacter baumannii* [111]. Diverse classes of efflux pump inhibitors were discovered from natural sources, in particular from plant [112,113]. Farnesol acts as an effective efflux pump inhibitor in *Mycobacterium smegmatis* [114]. Coumarins from *Mesua ferrea* has been established as an efflux pump inhibitor of NorA in *S. aureus* [115]. Phenyl propanoids isolated from *Alpina galangal* and a homoisoflavonoid, bonducellin from *Caesalpinia digyna* root function as EPI in *Mycobacterium smegmatis* [116,117]. Piperine was established as an effective efflux pump inhibitor of MdeA efflux pump of *S. aureus* and also

against a putative multi drug transporter in *M. tuberculosis* [118,119].Capsaicin (8-methyl-N-vanillyl-6-nonenamide) functions as an inhibitor of NorA in *S. aureus* [120].

3.7. Fluoroquinolone resistance in bacteria

Fluoroquinolones are clinically useful broad spectrum antibiotics used to treat infection caused by gram negative bacteria. The primary reason of fluoroquinolone resistance in gram negative bacteria is the mutation at quinolone resistance determining region (QRDRs) of *gyrA* encoding DNA gyrase A subunit and *parC* encoding the topoisomerase IV subunit [121,122]. However, fluoroquinolone resistant mutant of *A. baumannii* have been isolated which do not have mutation in those regions [123]. Fluoroquinolones are substrates for several efflux pumps which contribute fluoroquinolone resistance to the gram negative bacteria [124,125]. Efflux mediated fluoroquinolone resistance was also found in *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* [126]. Overexpression of *patAB* is also associated with fluoroquinolone resistance in various clinical isolates [127]. Expression of *patA* and *patB* encoding two ABC transporters in *Streptococcus pneumonia* were found to be induced by fluoroquinolones [128]. AbeM is the most significant efflux pump in *A. baumannii* which confers fluoroquinolone resistance to the organism [86]. High level of efflux mediated fluoroquinolone resistance was observed in *Mycobacterium smegmatis* [129]. An elevated expression ABC transporter genes were found in fluoroquinolone resistant mutant of *M. smegmatis* [130,131].

3.8. Identification and characterization of efflux pump inhibitor (EPI) against AbeM, a multidrug efflux pump in *Acinetobacter baumannii*

The next section of this chapter deals with detailed description of the study which was carried out for identification and characterization of novel efflux pump inhibitors against AbeM, a proton (H^+) driven multidrug efflux pump in *A. baumannii*.

3.8.1. *Materials*

3.8.1.1. Chemicals

The small molecule library was purchased from Maybridge (Trevillett, UK). All the antibiotics, substrate molecules and carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP) were purchased from Sigma-Aldrich (St. Louis, USA). Dimethyl sulphoxide (DMSO), Luria Bertani broth (LB) (Miller), Mueller Hinton (MH) broth and agar-agar were purchased from Merck (Darmstadt, Germany). Restriction enzymes, *pfu* polymerase, T4 ligase, ATP, dNTPs, alkaline

phosphatase, and other molecular biology grade chemicals were purchased from Thermo Fisher Scientific (Waltham, USA). 3-(4,5-dimethyl-2-thiazolyl)2,5diphenyl-2H-tetrazoliumbromide (MTT), agarose and other analytical grade chemicals were purchased from HiMedia (Mumbai, India).

3.8.1.2. Bacterial strains and primers

All the bacterial strains and primers used in this study are given in Table.3.1 and Table.3.2 respectively. All the strains were grown at 37°C, with 200 rpm agitation, in Mueller Hinton broth. Ampicillin was used at 100 mg/L whenever required. The clinical strains were confirmed by species specific PCR amplification, Gram staining and maintained on Leeds Acinetobacter Medium (LAM).

Table 3.1 List of the strains and organisms used in the study

Organism	Strain	Description	Reference
Acinetobacter baumannii	ATCC 17978	Reference strain of *A. baumannii*	Purchased from ATCC, USA
	AYE	Drug resistant strain of *A. baumannii*	Purchased from ATCC, USA
	RPTC-19	Clinical isolate expressing AbeM, exhibiting high resistance to fluoroquinolones	Obtained from Dr. V. Gupta, GMCH, Chandigarh
Escherichia coli	DH5α	Host strain used for maintaining plasmids	Purchased from Invitrogen, USA
	KAM32	A double knock out strain (Δ*ydhE*, Δ*acrB*) lacking major efflux pumps, sensitive to most of the antibiotics	Obtained from Dr. A. Bhardwaj, IIAR, Gandhinagar. Constructed by Chen *et al*.
	KAM32/pUC18	KAM32, carrying vector pUC18	This study
	KAM32/pUC18-*abeM*	KAM32, expressing *abeM* cloned in pUC18	This study

Table 3.2 List of the primes used in the study

Sr. No.	Primer	Sequence	Remarks
1	FPABEM	ACTGGAATTCCTTTACAGGG TAGTCGTTTTGG	For cloning *abeM*, with native promoters, in pUC18.
2	RPABEM	AGCTAAGCTTAGCCATTTTG CTGAATCCGAAG	For cloning *abeM*, with native promoters, in pUC18.
3	FPRTABEM	TGTAACCATGTATGCAGCGC	For amplification of an internal region of *abeM* ORF in RT-PCR reaction
4	RPRTABEM	ATGTTGCAAAACCACAGCCT	For amplification of an internal region of *abeM* ORF in RT-PCR reaction
5	FPRT16S	CAGCTCGTGTCGTGAGATGT	For amplification of an internal region of 16S rRNA coding gene in RT-PCR
6	RPRT16S	CGTAAGGGCCATGATGACTT	For amplification of an internal region of 16S rRNA coding gene in RT-PCR

3.8.2. *Primary screening of small molecule library for novel EPI against* AbeM

This study began with screening of a small molecule library of 8000 molecules for identifying a potential efflux pump inhibitor against AbeM, a multidrug efflux pump in *A. baumannii*. *E. coli* KAM32, a strain of *E. coli* devoid of major efflux pumps YdhE and AcrB has been utilised for screening (Table.3.1). This is a very sensitive strain of *E. coli* without any efflux activity of its own. The *abeM* locus from *Acinetobacter baumannii* was cloned into pUC18 and transformed into *E. coli* KAM32 cells to obtain *E. coli* KAM32/pUC18-*abeM*. Similarly pUC18 was transformed into *E. coli* KAM32 to obtain *E. coli* KAM32/ pUC18. Elevated resistance of *E. coli* KAM32/pUC18-*abeM* compared to *E. coli* KAM32/ pUC18 against ciprofloxacin (one of the substrate of AbeM) has been utilized for screening the library.

3.8.2.1. Cloning of abeM from A. baumannii in E. coli KAM32

The *abeM* (accession number AB204810) ORF along with its promoter and terminator was amplified using primers FPABEM and RPABEM (Table.3.2) from genomic DNA of *A. baumannii* ATCC 17978. The 1.9kb amplicon was double digested with EcoRI and HindIII and ligated with similarly digested pUC18 and transformed into *E. coli* DH5α. The recombinant plasmid (pUC18-*abeM*) obtained from *E. coli* DH5α was confirmed by restriction digestion and was transformed into *E. coli* KAM32 resulting in *E. coli* KMA32/pUC18-*abeM*. pUC18 alone was transformed into *E. coli* KAM32 to obtain *E. coli* KAM32/pUC18 that was used as control strain in the further experiments.

3.8.2.1.1. Method

Genomic DNA was isolated from *Acinetobacter baumannii* ATCC 17978 by using a genomic DNA isolation KIT (Thermo Fisher Scientific) according to manufacturer's instruction. The DNA was quantified by measuring its absorbance at 260 nm using a spectrophotometer. 1 µl of this DNA was used as a template for the amplification of *abeM.* The detail of PCR reaction mixture is given in Table.3.3. The program which has been optimized for the amplification of *abeM* is given in Table.3.4.

Table 3.3 PCR reaction (50 µl) for amplification of *abeM* from *A. baumannii*

Reagents	**Stock concentration**	**Final concentration**	**Volume (µl) used in 50 µl reaction**
Primer Forward	10 µM	0.4 µM	2
Primer Reverse	10 µM	0.4 µM	2
Pfu Buffer	10X	1X	5
dNTPs	2 mM	0.2 mM	5
Pfu Polymerase enzyme	2.5 U/µl	2.5 U	1
Template DNA	100 ng/µl	100 ng	1
Nuclease free water	-	-	34
		Total	**50 µl**

Table 3.4 PCR program used for the amplification of *abeM*

Steps	**Temperature**	**Duration**	**Cycles**
1	95°C	2 min	
2	95°C	45 sec	5 cycles
	55°C	45 sec	
	72°C	2 min	
3	95°C	45 sec	25 cycles
	57°C	45 sec	
	72°C	2 min	
4	72°C	10 min	
5	4°C	Hold	

Restriction digestion of the PCR amplified product and pUC18 was done according to Table.3.5 and 3.6. Reaction mixture was incubated at 37°C for 2 h in a dry bath in a microcentrifuge tube. Then the enzymes were heat inactivated by heating at 65°C for 20 min.

Table 3.5 Restriction digestion of the PCR amplified product

Reagents	Concentration	Volume (µl)
PCR amplified DNA	1.5 ng	35
10 X NEB Buffer2	1X	5
E.coR1(NEB)	20 U	1
HindIII (NEB)	20 U	1
Nuclease free water	-	8
	Total	**50 µl**

Table 3.6 Restriction digestion of vector plasmid pUC18

Reagents	Concentration	Volume (µl)
Plasmid DNA	1.5 ng	30
10 X NEB Buffer2	1X	5
EcoR1(NEB)	20 U	1
HindIII (NEB)	20 U	1
Nuclease free water	-	13
	Total	**50 µl**

Restriction digestion mixture was incubated at 37°C for 2 h in a dry bath in a microcentrifuge tube. Then the enzymes were heat inactivated by heating at 65°C for 20 min. The DNA was purified by using a PCR cleanup kit (Qiagen) and treated with, Calf intestinal alkaline phosphatase (CIP) for 1 h at 37°C to remove the terminal phosphates of the vector DNA (Table.3.7). The DNA was then again purified by using a mini elute kit (Qaigen). A ligation mixture was set up (Table.3.8) at 22°C for 2 h. The reaction was stopped by heating the tube in dry bath at 70°C for 5 min.

Table 3.7 Alkaline phosphatase treatment of double digested pUC18 vector DNA

Reagents	Volume (µl)
Digested vector DNA	35
NEB Buffer3	4
NEB CIP	1
Total	**40µl**

Table 3.8 Ligation mixture for digested pUC18 and *abeM* inserts

Reagents	Concentration	Volume (µl)
Insert DNA (*abeM*)	600 ng	11
Vector DNA (pUC18)	200 ng	5
10X T4 Ligase Buffer (Thermo Scientific)	1X	2
10 mM ATP	0.5 mM	1
T4 Ligase (Thermo Scientific)	10 U	1
	Total	**20 µl**

2 µl of ligated mixture was transformed into electro-competent *E. coli* DH5α cells prepared earlier by using an electroporation unit (Eppendorf) at pulse of 2000V. Transformed cells were incubated 37°C immediately in rich liquid media for an h in shaking condition. Cells were then plated on to a LB Agar plate with ampicillin (100 µg/ml) and incubated at 37°C overnight. Next morning, four well isolated colonies of the transformants were picked individually and inoculated into LB broth with ampicillin (100 µl). The culture tubes were incubated at 37°C, 200 rpm for 12 h. The plasmid DNA was isolated and restriction digestion was carried out for confirming the cloning of *abeM* into pUC18. Recombinant plasmid, pUC18-*abeM* and pUC18 were transformed in two different *E. coli* KAM 32 cells by electroporation to produce *E. coli* KAM32/pUC18-*abeM* (test strain) and *E. coli* KAM32/pUC18 (control strain). The *E. coli* KAM32/pUC18-*abeM* construct was also confirmed by sequencing (section 3.8.2.2).

3.8.2.1.2. Result

The cloning of *abeM* in pUC18 (Fig.3.2A) was confirmed by the restriction digestion of the recombinant plasmid (pUC18-*abeM*) by EcoR1 and HindIII (Fig.3.2B).

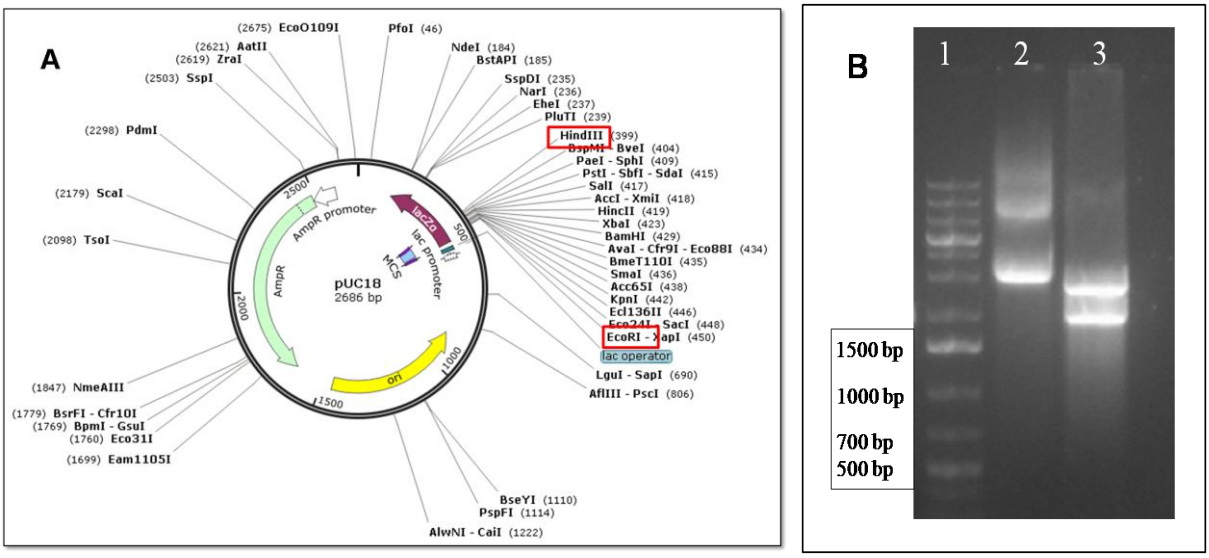


Figure 3.2 (A) Plasmid map of pUC18. Red squares indicating the restriction sites HindIII and EcoRI **(B)** 1% Agarose gel picture of a restriction digestion of plasmid pUC18-*abeM* confirming the cloning of *abeM* into pUC18. Double digestion of the plasmid pUC18-*abeM* (lane2) with EcoRI and HindIII resulted in the release of 1.9 kb fragment of *abeM* (lane 3). Lane 1 was a 1 kb ladder.

3.8.2.2. Sequencing of abeM

Sequencing was carried out by using the forward and reverse primer used for cloning of *abeM* from *A. baumannii* (given in Table.2). A sequence of 1261 base was obtained by the forward primer and a sequence of 1260 base was obtained from reverse primer. Two sequences were aligned to get the entire 1876 base sequence with a common region flanked by additional sequences. The entire sequence was identical with the sequence of *abeM* (accession number AB204810).Sequence obtained by the forward and reverse primer and their respective chromatograms (Fig.3.3A and 3.3B) are given in the following section 3.8.2.2.1.

3.8.2.2.1. Result

Sequence amplified by forward primer

>0413_029_001_AbeM_Clone_Primer-Forward-H11.ab1

CATTATTCTGATTGGTCTTTCTATGTTACTTGACCATTTTGGCTGGTTAGGTACCGA
AAAGACACCTGTTACATGGCAACGGTTTCTAGGTGGAGTGGTCATCTTTGTTGGTG
TATTACTTACCTTGCAACGCTAGTTTCATTTTTTATATTTATAAAGAGTACCTTTTGT
GTCGAATGTCACGTCGTTTCGGTCTGAATTAAAACAACTCTTCCATTTAATGTTACC
TATTTTAATTACGCATTTTGCTCAAGCAGGGTTTGGGTTAATTGATACCATTATGGC
TGGGCATTTATCTGCCGCAGACTTAGCCGCTATTGCGGTAGGTGTAGGCTTATGGA
TTCCAGTCATGCTCTTGTTCAGTGGCATAATGATTGCAACCACACCATTAGTTGCCG
AAGCAAAAGGCGCTAGAAATACAGAGCAAATTCCAGTGATTGTCCGCCAATCATT
ATGGGTTGCAGTAATTCTAGGGGTATTGGCAATGCTCATTTTGCAGCTTATGCCATT
TTTCTTACATGTGTTTGGCGTACCAGAAAGTTTACAACCTAAAGCCAGTTTATTCTT
ACATGCAATTGGTTTGGGTATGCCCGCTGTAACCATGTATGCAGCGCTCCGAGGCT
ATTCCGAAGCATTAGGCCATCCCCGTCCTGTCACGGTCATTAGCTTACTAGCCTTA
GTGGTTTTAATCCCGCTTAACATGATTTTTATGTATGGCTTAGGACCAATACCTGCT
TTGGGTAGCGCAGGCTGTGGTTTTGCAACATCCATTTTACAGTGGCTGATGCTCATT
ACGTTAGCAGGCTATATTTATAAGGGCTTCGGCTTATCGAAACACATCTATTTTTA
GCAGATTCGATAAAATTAATCTGACTTGGGTTAAAAGAATTTTACAGCTCGGTCTG
CCAATTGGTTTAGCTGTGTTTTTTGAAGTGAGTATTTTTAGTACAGGGGCATTGGTC
CTTAGCCCTCTAGGGGAAGTCTTTATTGCCGCACACAAGTAGCGATTTCAGTCACT
TCGTACTGTTTATGATTCACTTTCTCTTGCATTGCTTTAACATTCGCGTGGACGTATT
ATGCGAAAAGAACTGGGCTCATGTATCAAGTACAAAATGTCTAAGCACAAGCAGT
ATTTTTTTGCTCTATTGACATGTCTTTTATTGCTTAGCGTTACATGGTCTCGGTTATA
CTCAAGATATAAGTTGGCGGTCAGTATTGCTGTTGCAATGACATTCATTATGGGAT
GGTCTCTTACAAAGCT

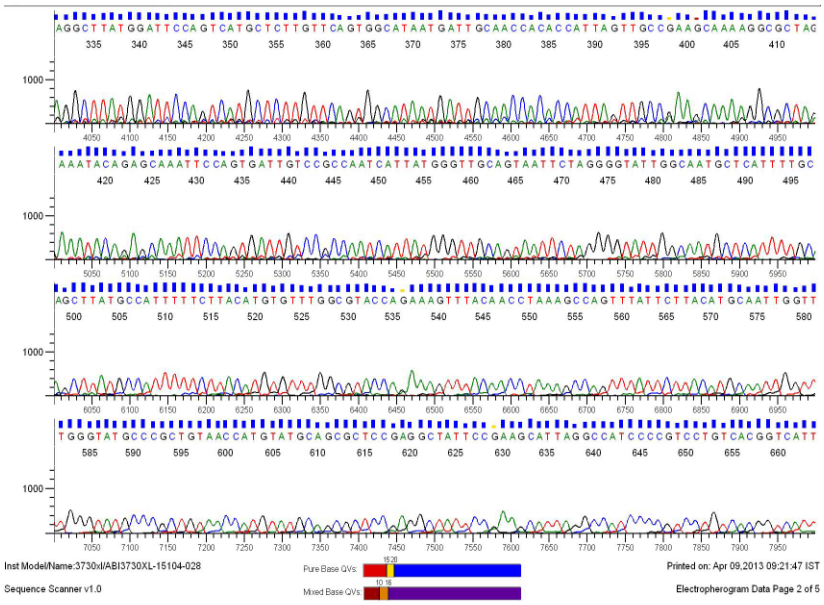


Figure 3.3 (A) Chromatogram of the sequencing by the forward primer.

Sequence amplified by reverse primer

>0413_029_002_AbeM_Clone_Primer-Reverse-A12.ab1

AGGAGTTTTTAAAGGAGAATCATTCATGTAGATGCCGAGCTTGAGTCGGCACAAG
GGCAGGTGGTGATTTCTAATCTAGTACTCGATTTAACAAGCCAGTTATTTAATGCC
TTAGGGGCATCGGCGAGTAGCCAAGTGAAACAACTTGACCGTTTCTGGCGTAATGC
TCGGACAGTGTCTTCACATAATCCGCTTATTTACAAAGAAAAAGTAATTGGAGATT
GGGAAGTAAACCGTACGGATTTACCATTTGTCTGGCAAATTGGAGCAAGCCCACG
GGCAAAGACTGCATAAAATAAAAAAAGCCGATGTTTCATCGGCTTTTTTATTAGGT
TTGACTTAAACGTTTGGTATTCAAATAGAGTCGTGAGAGCAATAAAACACAGGCA
ATACTTAAACCAATAATTAAACCTAACCACACACCAGCTACGCCCCAATCGGTATA
ACGCGCTAAATAAAGACCGATTGGAAAAGCAATTACCCAATACGCCATTAAGGTG
ATCCACATCGGTGCCTGAGTATCTTGCATACCTCTTAAACAGCCGGCAGCGCTGAC
TTGTAGAGCATCCATTAATTGATATGCCATTGCAAACCAGAGCAAATACATGGCAA
CCGGCACAACATTTATATCTTGAGTATAAACCGAGACAATTTGTTCACGGCCTAAA
GCAATAAAAGACATGGTCAATAGAGCAAAAAATACTGCTGTGCTTAGACCAATTT
TTTGTACTTGATACATGGAAGCCCAGTTCTTTTCGCCATAATACGTCCCCACGCGA
ATGGTTAAAGCAATGGCAAGAGAAAGTGGAATCATAAACAGTACCGAAGTGACTG
AAATCGCTACTTGGTGTGCGGCAATAAAGACTTCCCCTAGAGGGCTAGGACCAAT
GCCCCTGTACTAAAAATACTCACTTCAAAAACACAGCTAAACCAAGTTGGCAGAC
CGAGCTGTAAAATTCTTTTAACCCAAGTCAGATTAATTTTATCGAATCTGCTAAAA
ATAGATGTGTTTCGATAAGCGAAGCTTATAAATATAGCCTGCTACGTAATGAGCAT
CAGCACTGTAAAATGGATGTTGCAAACACAGCTGCGCTACCCAAAGCAGGTATTG
GTCTAGCATACATAAAAATCATGTTAGCGGGATTAAACACTAGCTAGTAGCTATTG
ACCGTGAGGGACGGATGCTATGTCTTCGAAGCCCTGAGCGTCTAACTTGGTACGCG
GCTATCACCATGCAGGTAGAATTAACTGGCCTTTAAG

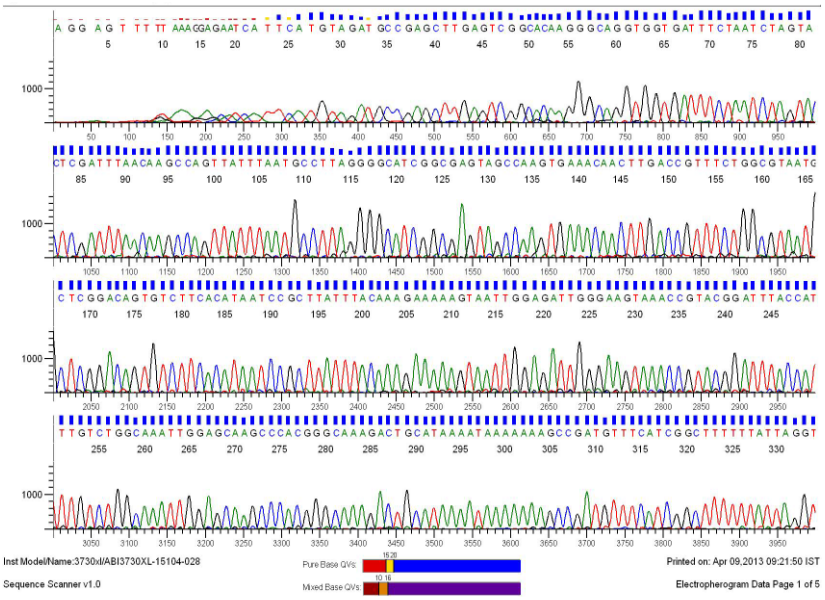


Figure 3.3 (B) Chromatogram of the sequencing by the reverse primer.

3.8.2.3. Determination of MICs of various substrates of AbeM against *E. coli* KAM32/pUC18-*abeM* as compared to *E. coli* KAM32/pUC18

3.8.2.3.1. Method

The Minimum Inhibitory Concentrations (MICs) of various antibiotics and antibacterial agents which are substrates of AbeM were determined against both *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18-*abeM* by the broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines in 96 well microtitre plate in Muller Hinton broth as described in section 2.13.1.

3.8.2.3.2. Result

The MICs of various substrates of AbeM against both *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18-*abeM* are given in Table.3.9. It was observed that *E. coli* KM32/pUC18-*abeM* exhibited elevated resistance to various substrates of AbeM including ciprofloxacin and norfloxacin. The *E. coli* KM32/pUC18-*abeM* was 32 fold more resistant to ciprofloxacin in comparison to *E. coli* KM32/pUC18 (control). The elevated resistance of *E. coli* KM32/pUC18-*abeM* for ciprofloxacin has been utilized for primary screening.

Table 3.9 Relative fold change in MIC of different drugs against *E. coli* KAM32/pUC18-*abeM* as compared to *E. coli* KAM32/pUC18.

Antibacterial Compounds	MIC in (mg/L)		Fold increase in MIC (Relative resistance)
	E. coli KAM32/pUC18	*E. coli* KAM32/pUC18-AbeM	
Ciprofloxacin	0.0015	0.05	32
Norfloxacin	0.01	0.3125	32
Ofloxacin	0.01	0.04	4
Erythromycin	0.63	1.25	2
Chlorohexidin	0.31	0.625	2
Clindamycin	1.25	1.25	1
Chloramphenicol	0.23	0.5	2
Kanamycin	2.5	2.5	1
Gentamycin	2.5	5	2
Tetracycline	0.08	0.16	2
Rifampin	2.5	2.5	1
Trimethoprim	0.02	0.02	1
DAPI	0.39	3.125	8
Hoechst 33342	0.31	1.25	4
TPPCl	1.25	5	4
Acriflavin	3.13	6.25	2
EtBr	3.13	6.25	4

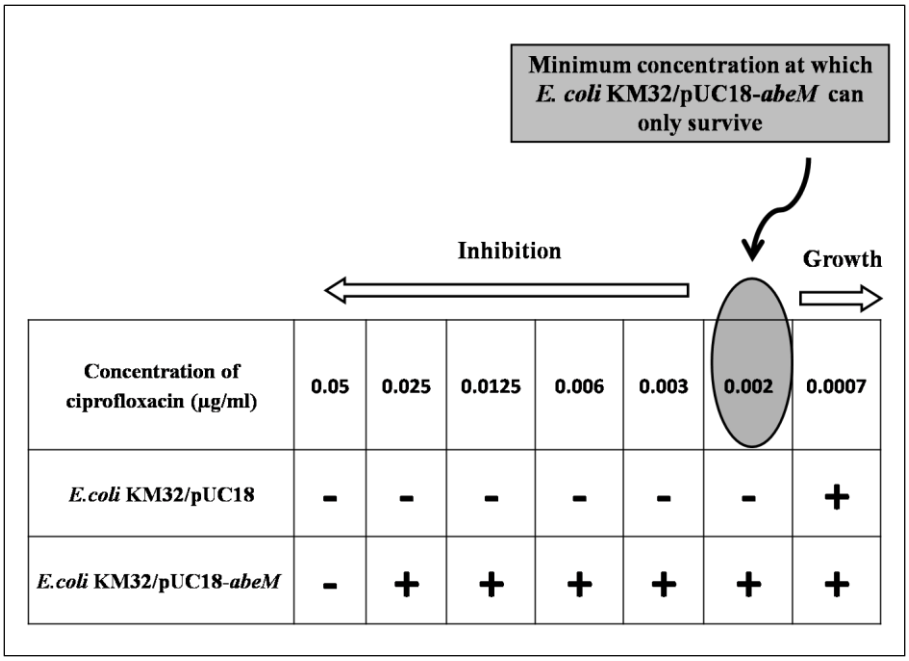
3.8.2.4. Determining a threshold minimum concentration of ciprofloxacin for primary screening assay

3.8.2.4.1. Method

A minimum threshold concentration of ciprofloxacin was determined against *E. coli* KAM32/pUC18-*abeM* for primary screening assay. This concentration was the minimum possible concentration of ciprofloxacin at which *E. coli* KAM32/pUC18-*abeM* can readily grow but *E. coli* KAM32/pUC18 cannot survive. This concentration of ciprofloxacin does not affect the growth of *E. coli* KAM32/pUC18-*abeM*, but does not allow the *E. coli* KAM32/pUC18 to grow. Both the control and the test strains were grown several times in presence of that threshold minimum concentration to ensure that only the strain with recombinant plasmid can grow at that concentration.

3.8.2.4.2. Result

A schematic of determination of threshold minimum concentration is given in Fig.3.4. It was observed that at a concentration of 0.002 μg/ml of ciprofloxacin *E. coli* KAM32/pUC18-*abeM* could readily grow but the growth of *E. coli* KAM32/pUC18 was inhibited. Therefore, 0.002 μg/ml of ciprofloxacin was determined as minimum threshold concentration.



Concentration of ciprofloxacin (μg/ml)	0.05	0.025	0.0125	0.006	0.003	0.002	0.0007
E.coli KM32/pUC18	-	-	-	-	-	-	+
E.coli KM32/pUC18-*abeM*	-	+	+	+	+	+	+

Figure 3.4 Determination of threshold minimum concentration of ciprofloxacin for primary screening: The minimum concentration of ciprofloxacin (marked by arrowhead) at which only *E. coli* KAM32/pUC18-*abeM* was able to survive was selected as threshold minimum concentration of ciprofloxacin for primary screening.

3.8.2.5. Primary Screening Assay

For primary screening, *E. coli* KAM32/pUC18-*abeM* was simultaneously grown in presence of 25 μM of small molecules (control) and in presence of 25 μM small molecules with 0.002 μg/ml of ciprofloxacin (Test) (Fig.3.5).Growth in both the conditions were monitored by measuring the absorbance of the culture at 600 nm to select probable efflux pump inhibitor. The molecules which did not inhibit the growth of *E. coli* KAM32/pUC18-*abeM* by its own at 25 μM (in control plate) but inhibited the growth in combination with an ineffective concentration (0.002μg/ml) of ciprofloxacin (in test plate) were selected as a primary lead.

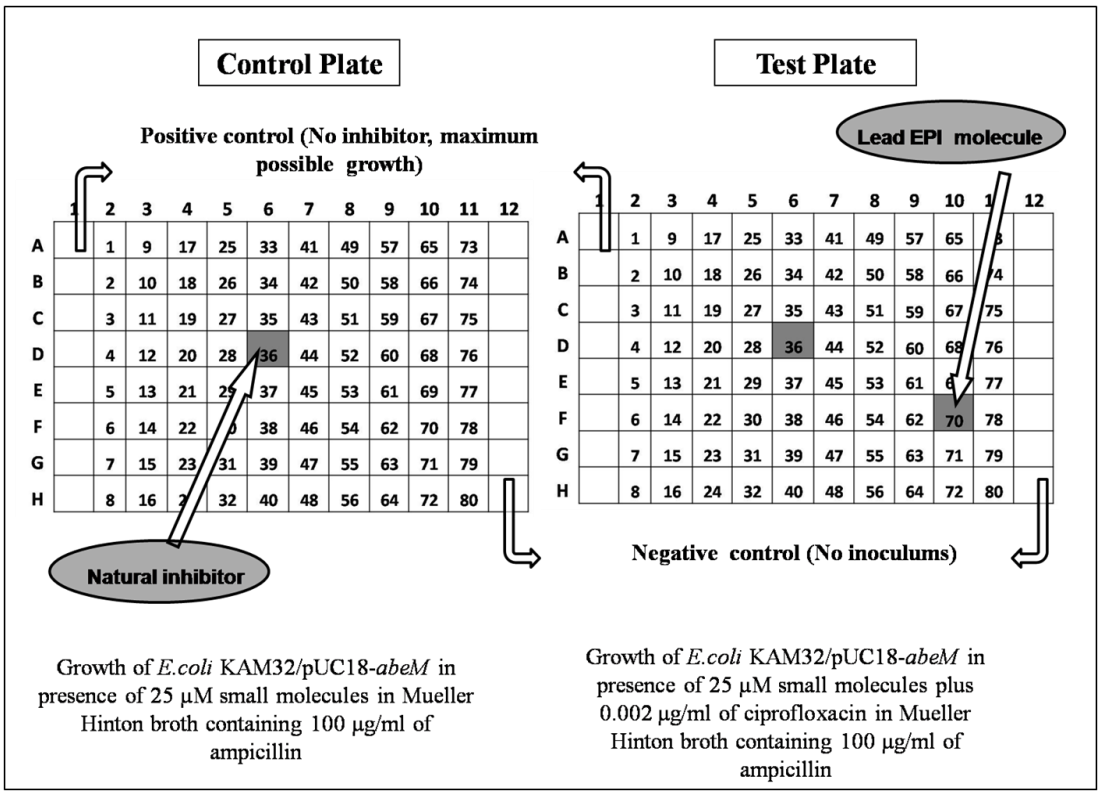


Figure 3.5 Schematic representation of primary screening assay: 80 molecules were screened in two 96-well plates. In one plate (control plate), *E. coli* KAM32/pUC18-*abeM* was grown in presence of only 25 μM of small molecules. In another plate (Test plate), *E. coli* KAM32/pUC18-*abeM* was grown in presence of combination of small molecules (25μM) and ciprofloxacin (0.002 μg/ml, threshold minimum concentration). First column (A1-H1) in each plate was kept as positive control where no antibiotic or small molecules were added to the media. Organisms were only grown in the media with 1% DMSO (solvent). Last column (A12-H12) in each plate was considered as negative control where no inoculum was added. Growth in the positive control was maximum and was considered as 100% growth. There was no growth in negative control. Well no. D6 is exhibiting inhibition in both the plates, indicating compound no 36 is a inhibitor of growth. Well no. F10 exhibit inhibition in growth only in test plate, indicating compound no 70 is a potential lead EPI. In the second case, an ineffective concentration (0.002 μg/ml) of ciprofloxacin inhibiting the growth of *E. coli* KAM32/pUC18-*abeM* in combination with 25 μM of small molecule which itself is not inhibitory for the cells.

3.8.2.5.1. Method

1. A set of two 96-well microtitre plates were used for screening each set of 80 molecules (Fig.3.5). All the molecules were tested at a concentration of 25 μM in same arrangement as they were archived in storage plate.
2. Muller Hinton broth supplemented with ampicillin (100 μg/ml) was used throughout the screening.
3. Fresh culture in liquid media having OD of 0.5 at 600 nm was diluted 10^3 fold and 100 μl of diluted culture was used as inoculum in the assay.
4. 2 μl of small molecules from 2.5 mM stock was added to all the wells in **control plate** except first (A1-H1) and last (A12-H12) columns.
5. All the wells except A1-H1 and A12-H12 had both antibiotic (ciprofloxacin) and small molecules in combination. Media was supplemented with (2 x 0.002 μg/ml) concentration of ciprofloxacin before putting the assay. 2 μl of small molecules from 2.5 mM stock was used in the test plates also. The final concentration of ciprofloxacin after adding 100 μl of inoculums was 0.002 μg/ml and the small molecule concentration was 25 μM.
6. There were two controls in each plate. First column in each plate (A1-H1) in every plate were kept as positive control where no antibiotics or small molecules were added. Test strain was only grown in presence of 1% DMSO.
7. Last column in each plate (A12-H12) were kept as a negative control where no inoculum was added.
8. The plates were incubated at 37°C in a humidity controlled incubator (Khuner Shaker X) for 24 h.
9. The growth was measured in terms of OD at 600 nm by using a plate reader (SpectraMax M2e Plus).
10. The percentage growth for each molecule was calculated in comparison to the positive control where no small molecule or antibiotic were added.
11. The average growth in the positive control was considered as 100% growth.
12. Percentage growth was calculated by using the following formula

Percentage Growth =(OD_{600} at Test well / OD_{600} at Positive control) X 100

13. The molecules which exhibit growth inhibition in only Test plate were considered as primary leads.
14. Molecules which exhibit inhibition in both the plates were not considered for secondary screening as they were growth inhibitors themselves.

15. A scatter plot was drawn by using GraphPad Prism software with percentage growth in presence of small molecule at X axis (control plate) and percentage growth in presence of ciprofloxacin combined with small molecule at Y axis (test plate) .

3.8.2.5.2. Results

The scatter plot of primary screening of 8000 small molecules against *E. coli* KAM32/pUC18-*abeM* is given in Fig.3.6. Percentage growth of control was plotted along the X-axis and percentage growth of test was plotted along the Y-axis. The molecules which alone could not inhibit the growth of *E. coli* KAM32/pUC18-*abeM* (control plate) but enabled ciprofloxacin to inhibit the growth (test plate) were picked for the secondary screening. Therefore, molecules which were effective when used in combination with an ineffective concentration (0.002μg/ml) of ciprofloxacin were selected as primary leads. Two ineffective concentrations (small molecules and ciprofloxacin) became effective when used in combination. A group of 28 molecules were selected after the primary screening (Table.3.10). The percentage growth for primary leads along the X-axis was close to 100 (no inhibition). In contrast growth percentage along the Y axis was zero (complete inhibition).

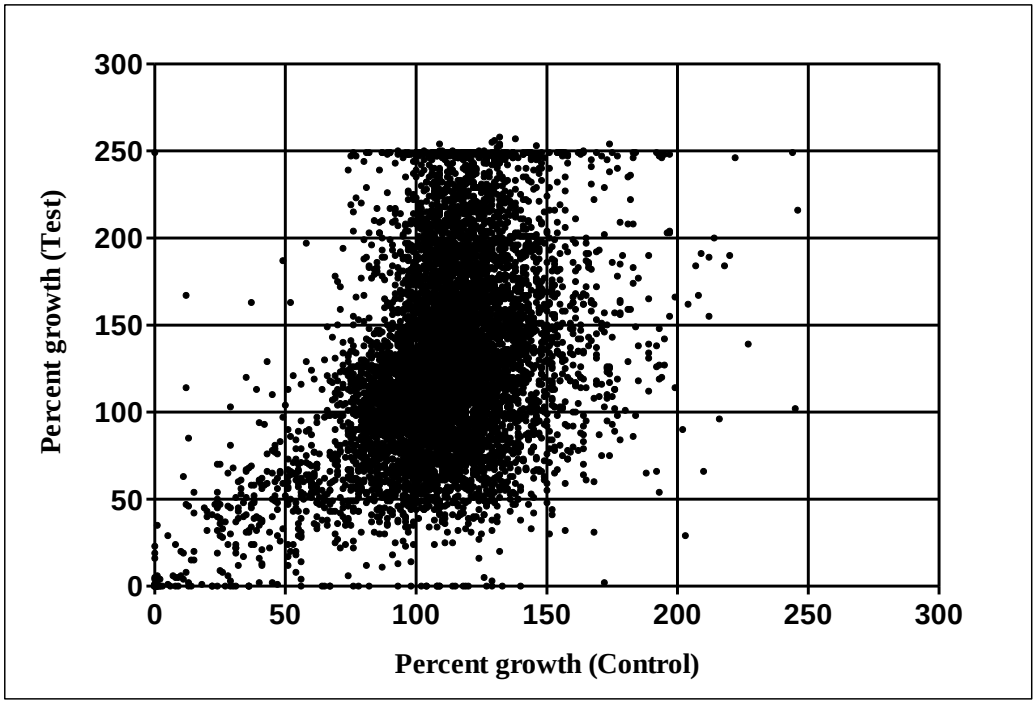


Figure 3.6 Scatter plot of primary screening of 8000 small molecules against *E. coli* KAM32/pUC18-*abeM*: Percentage growths of the recombinant strain in presence of only 25 μM of small molecules (control plate) were plotted along the X-axis. Percentage growths of the recombinant strain in presence of ciprofloxacin (0.002 μg/ml) in combination with 25 μM of small molecules (test plate) were plotted along the Y-axis.

Table 3.10 List of the 28 lead molecules found after the primary screening. The molecules which did not inhibit the bacterial growth by its own (along the X axis) but inhibit the growth in combination with an ineffective concentration of ciprofloxacin (along the Y axis) were selected.

Sr. No	Primary lead molecules	Growth percentage	
		Control (X axis)	Test (Y axis)
1	IITR05822	119	0
2	IITR06069	109	0
3	IITR06331	115	0
4	IITR06382	140	0
5	IITR06474	108	0
6	IITR07034	127	0
7	IITR07042	120	0
8	IITR07066	109	0
9	IIITR07408	114	0
10	IITR07784	65	0
11	IITR08027	126	5
12	IITR08367	67	0
13	IITR08678	67	0
14	IITR08913	78	0
15	IITR09576	104	0
16	IITR09804	133	0
17	IITR09830	118	0
18	IITR09838	93	0
19	IITR09905	172	2
20	IITR09990	108	0
21	IITR10025	129	0
22	IITR10035	104	0
23	IITR10133	82	0
24	IITR10156	76	0
25	IITR10275	129	3
26	IITR10658	98	0
27	IITR10677	102	0
28	IITR10785	56	0

3.8.3. Secondary Screening

28 molecules that have been selected in primary screening were tested at secondary level for their efficiency. Three assays based on antibiotic susceptibility were carried out in a stepwise manner to select best lead molecules.

3.8.3.1. Determination of MICs of ciprofloxacin and norfloxacin in combination with 28 lead molecules

3.8.3.1.1. Method

Ciprofloxacin and norfloxacin are two very important substrates of AbeM. The MICs of these two antibiotics in combination with 28 lead molecules individually were determined in 96-well plates in liquid media for selecting best molecules which can potentiate the activity of these antibiotics against *E. coli* KAM32/pUC18-*abeM*. 250 μM of the lead molecules were added in the 12th well in combination with the antibiotics and serially diluted along with the antibiotics for determining the MIC in a similar manner as described in section 2.13.1. MICs of these two antibiotics in combination of 28 primary lead molecules were compared. Carbonyl cyanide 3- chlorophenylhydrazone (CCCP), a known inhibitor was taken as control and also used in combination with the antibiotics. Molecules which exhibited significant fold of reduction in MICs of both antibiotics were tested at further.

3.8.3.1.2. Result

Out of 28 primary leads 13 molecules exhibited decrease in MICs for both the antibiotics when used in combination (Fig.3.7 and 3.8). These 13 molecules were tested at next level.

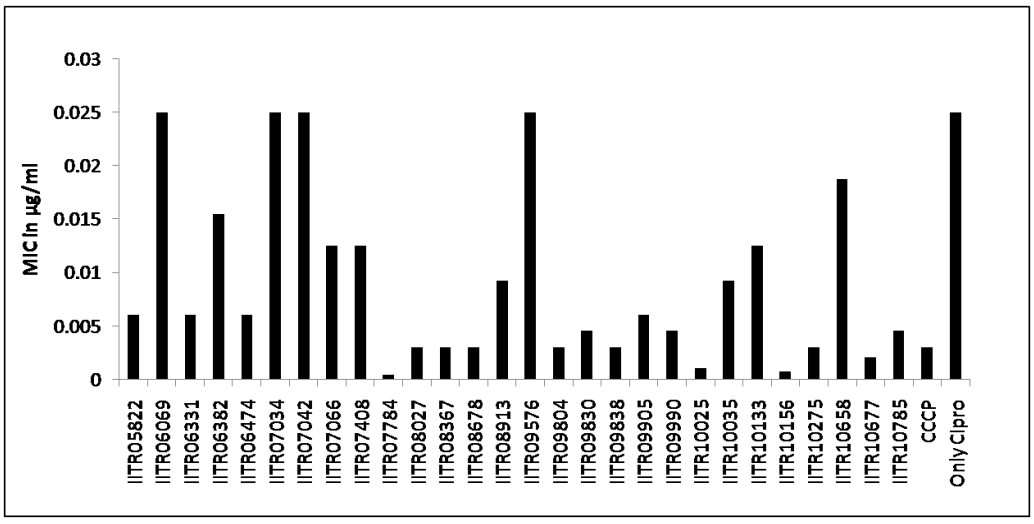


Figure 3.7 MIC of Ciprofloxacin in combination with primary lead molecules.

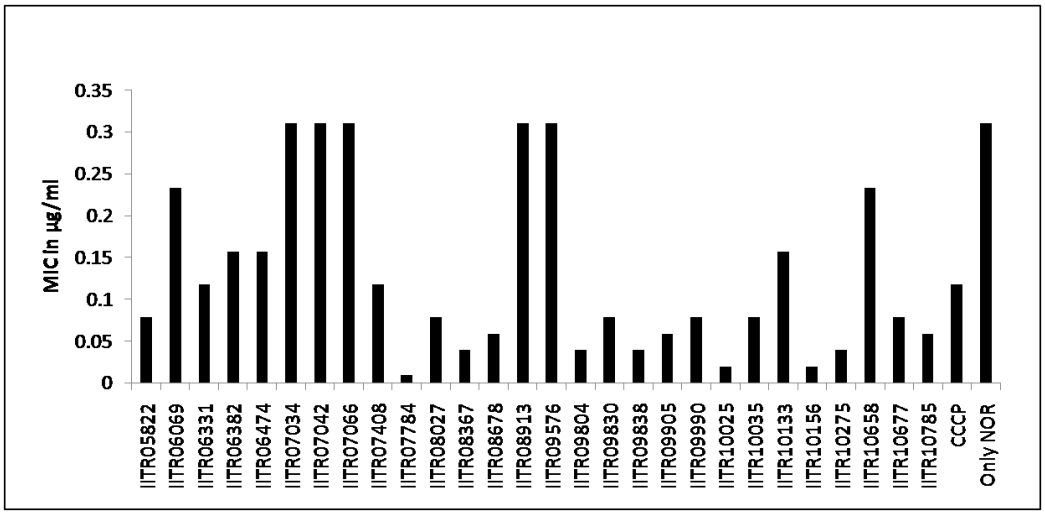


Figure 3.8 MIC of Norfloxacin in combination with primary lead molecules.

3.8.3.2. Determination of MICs of ciprofloxacin, norfloxacin and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) in presence of selected 13 lead molecules

3.8.3.2.1. Method

4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI), a fluorescent dye is also a major substrate of MATE family of pumps. The MICs of ciprofloxacin, norfloxacin and DAPI were determined in 96-well plate in presence and absence of 50 µM of 13 lead molecules which have been selected previously in section 3.8.3.1.2. In this assay, liquid media (Mueller Hinton broth) was supplemented with 50 µM of lead molecules before setting up the plates. MIC was determined as described in section 2.13.1. The concentration of lead molecules were kept constant (50 µM) in all the wells. In the previous section 3.8.3.1, lead molecules were used in combination with the antibiotics. Therefore, lead molecules were also getting diluted along with the antibiotics. Determining MICs in presence of a constant amount (50 µM) of small molecule require larger amount of small molecule. Therefore, a preliminary assay was carried out to exclude those molecules which did not exhibit significant reduction in MICs in combination with both the antibiotics. Only 13 molecules which exhibited good activity were tested in this section 3.8.3.2.

3.8.3.2.2. Result

8 molecules out of 13 exhibited a significant reversal in the MIC of ciprofloxacin, norfloxacin and DAPI against *E. coli* KAM32/pUC18-*abeM* (Table.3.11). These 8 molecules were tested further with 18 different substrates of AbeM in section 3.8.3.3

Table 3.11 MIC of ciprofloxacin, norfloxacin and DAPI in presence of 50 μM of lead molecules.

Sr. No		MIC in μg/ml			Fold decrease in MIC		
		Ciprofloxacin	**Norfloxacin**	**DAPI**	**Ciprofloxacin**	**Norfloxacin**	**DAPI**
**	**Control**	0.05	0.31	1.5	1	1	1
1	IITR05822	0.003	0.019	0.187	16	16	8
2	IITR06382	0.025	0.155	0.75	2	2	2
3	IITR06474	0.006	0.019	0.375	8	16	4
4	IITR07034	0.025	0.155	0.75	2	2	2
5	IITR07042	0.05	0.31	1.5	1	1	1
6	IITR07066	0.012	0.019	0.375	4	16	4
7	IITR08027	0.002	0.01	0.098	25	31	15
8	IITR08678	0.003	0.01	0.187	16	32	8
9	IITR09804	0.003	0.01	0.093	16	32	16
10	IITR09990	0.003	0.02	0.098	17	16	15
11	IITR10025	0.003	0.01	0.093	16	32	16
12	IITR10658	0.012	0.155	0.75	4	2	2
13	IITR10677	0.003	0.02	0.195	17	16	8

** MIC alone (without any small molecule) was considered as **control**.

3.8.3.3. Determining the MICs of 18 different substrates of AbeM in presence of 8 lead molecules at concentration of 50 μM

3.8.3.3.1. Method

Out of 13 molecules, 8 molecules were selected and tested further to select best molecule capable of potentiating the maximum number of substrates of AbeM. MIC of 18 substrates belonging to diverse chemical classes was determined in presence of 8 lead molecules at a concentration of 50 μM in a similar fashion as described in section 3.8.3.2

3.8.3.3.2. Result

All the 8 molecules exhibited significant decrease in MICs of number of substrates including ciprofloxacin and norfloxacin (Table.3.12 and 3.13). The result suggests that these molecules inhibit AbeM and enhance the efficiency of its substrates. All these molecules are

potential efflux pump inhibitors (EPIs) of AbeM and need to be studied further for their detailed characterization.

Table 3.12 MIC of 18 different substrates antibacterial agents in presence of 8 lead molecules.

Sr. No	Antibacterial agents	MIC in Absence of EPIs (µg/ml)	MIC in Presence of 50 µM EPIs (µg/ml)							
			IITR05822	IITR06474	IITR08027	IITR08678	IITR09804	IITR09990	IITR10025	IITR10677
1	Ciprofloxacin	0.05	0.0031	0.05	0.0031	0.0016	0.0031	0.0063	0.0031	0.0031
2	Norfloxacin	0.625	0.0098	0.625	0.0098	0.0049	0.0098	0.019	0.019	0.078
3	Ofloxacin	0.0313	0.0039	0.0313	0.0039	0.0039	0.0078	0.0156	0.0078	0.002
6	Erythromycin	0.625	0.625	0.625	0.625	0.078	0.078	0.625	0.31	0.625
4	Chlorohexidin	0.625	0.078	0.625	0.0098	0.019	0.078	0.156	0.078	0.625
5	Phosphomycin	3.125	0.78	3.125	0.195	0.39	0.097	0.78	0.78	1.56
7	Clindamycin	2.5	0.3125	2.5	0.625	0.15	0.625	1.25	1.25	1.25
8	Chloramphenicol	0.937	0.234	0.937	0.468	0.23	0.234	0.468	0.468	0.468
9	Kanamycin	1.56	0.39	1.56	0.39	0.39	0.39	0.78	0.78	1.56
10	Gentamycin	0.39	0.195	0.39	0.19	0.39	0.19	0.39	0.39	0.39
11	Tetracyclin	0.78	0.097	0.78	0.78	0.39	0.78	0.39	0.39	0.39
12	Rifampin	5	1.25	5	2.5	0.625	1.25	2.5	2.5	2.5
13	Trimethoprim	0.0625	0.0313	0.5	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625
14	DAPI	1.56	0.097	1.56	0.097	0.048	0.024	0.39	0.19	0.39
15	Hoechst 33342	0.625	0.0196	0.625	0.019	0.0098	0.0195	0.625	0.625	0.31
16	TPPCl	5	1.25	5	0.625	0.31	0.625	5	5	5
17	Acriflavin	3.12	0.78	3.12	1.56	0.195	0.78	1.56	0.78	1.56
18	EtBr	6.25	1.56	6.25	1.56	0.195	0.78	1.56	0.78	1.56

Table 3.13 Fold decrease in MIC of 18 different antibacterial molecules in presence of 8 lead molecules.

Sr. No	Antibacterial agents	Fold Decrease in MIC in presence of 50 µM EPIs							
		IITR05822	IITR06474	IITR08027	IITR08678	IITR09804	IITR09990	IITR10025	IITR10677
1	Ciprofloxacin	16	8	16	32	16	8	16	16
2	Norfloxacin	64	2	64	128	64	32	32	8
3	Ofloxacin	8	1	8	8	4	2	4	16
6	Erythromycin	1	4	1	8	8	1	2	1
4	Chlorohexidin	8	8	64	32	8	4	8	1
5	Phosphomycin	4	4	16	8	32	4	4	2
7	Clindamycin	8	4	4	16	4	2	2	2
8	Chloramphenicol	4	2	2	4	4	2	2	2
9	Kanamycin	4	2	4	4	4	2	2	1
10	Gentamycin	2	2	2	1	2	1	1	1
11	Tetracyclin	8	2	1	2	1	2	2	2
12	Rifampin	4	4	2	8	4	2	2	2
13	Trimethoprim	2	1	1	1	4	1	1	1
14	DAPI	16	2	16	32	64	4	8	4
15	Hoechst 33342	32	2	32	64	32	1	1	2
16	TPPCl	4	4	8	16	8	1	1	1
17	Acriflavin	4	4	2	16	4	2	4	2
18	EtBr	4	4	4	32	8	4	8	4

3.8.3.4. Checkerboard titration assay

Interactions between ciprofloxacin and norfloxacin with 8 potential lead EPI molecules were assessed by checkerboard titration assay against *E. coli KAM32*/pUC18-*abeM* as described previously [132]. A schematic of checker board assay in 96-well plate is shown in the Fig.3.9. The grey region indicates inhibition. Ciprofloxacin were tested at 10 concentrations

(0.0002 to 0.1 µg/ml), while IITR molecules were tested at 8 concentrations (3 to 250 µM). The small molecules were dispensed alone in the first row (A2 to A10) and ciprofloxacin alone was dispensed alone in the first column (B2 to H2). The concentrations of the small molecules were diluted vertically from A to H rows and the concentration of ciprofloxacin was diluted horizontally from 1 to 10 columns. A2 to A10 (first row) contain highest concentration of the small molecule (500 µM) alone whereas B1 to H1 (first column) contain highest concentration of ciprofloxacin (0.1 µg/ml) alone. A1 well was kept as a control with no antibiotics or small molecules were added in that. As the small molecules and ciprofloxacin were diluted vertically and horizontally, various combinations were produced.

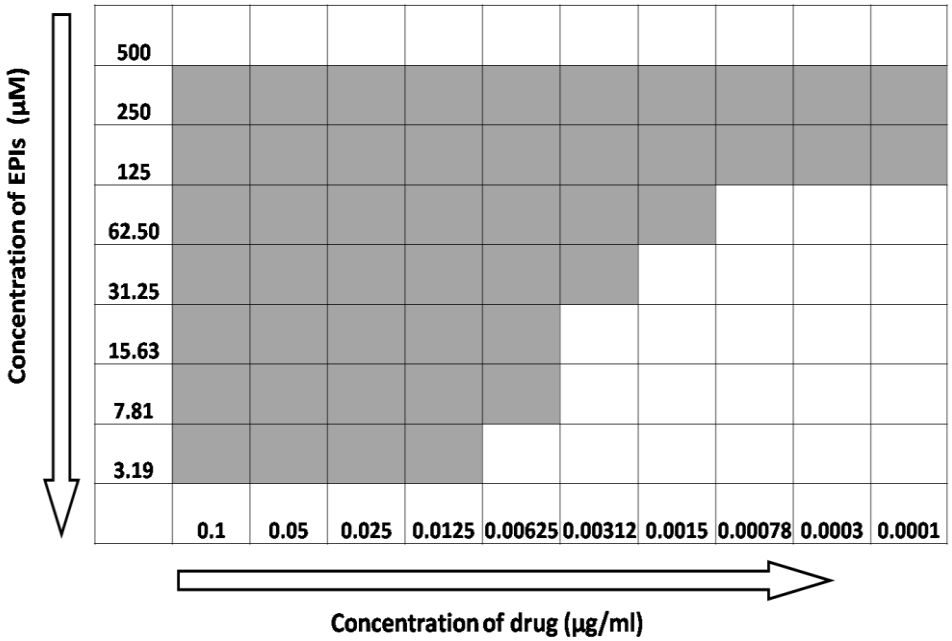


Figure 3.9 Schematics of checkerboard assay to establish synergy between efflux pump inhibitors and the drug.

The drug and small molecule interactions were classified as synergistic if the fractional inhibitory concentration (ΣFIC) index was less than 0.5. FIC index is the sum total of FIC of small molecule as well as the drug. FIC is the measure of MIC in combination divided by the MIC alone.

FIC of Antibiotics = MIC of Antibiotic in combination/ MIC of Antibiotic alone

FIC of EPI = MIC of EPI in combination / MIC of EPI alone

FIC index (ΣFIC) = FIC of EPI + FIC of Antibiotics

ΣFIC ≤ 0.5, indicates Synergy

3.8.3.4.1. Method

1. All the wells of the 96-well plates were filled with 100 µl of MH media with ampicillin (100 µg/ml). Same media was used throughout the experiments.
2. 96 µl of media was poured into all the wells from A1-H1 (first column of the 96 well plate)
3. 96 96 µl of media was poured into all the wells from A2-A10 (first row of the 96 well plate)
4. 4 µl of ciprofloxacin from 0.1 mg/ml stock was added to B1-H1
5. 4 µl of small molecule from 50 mM stock was added to A2-A10
6. 100 µl was transferred from B1-H1 to next column B2-H2. Again 100 µl was transferred from B2-H2 to next column B3-H3 by using multichannel pipette. In a similar fashion 100 µl was transferred up to B10-H10 (10^{th} column).
7. At this point, 100 µl of media was discarded from B10-H10 wells.
8. In this way, antibiotic was serially diluted horizontally from B1-H1 to B10-H10.
9. Similarly, small molecule was serially diluted from A2-A10 to H2-H10 vertically. After reaching at H2-H10, 100 µl media with small molecules and antibiotic was discarded.
10. Now, fresh culture with OD 0.5 at 600 nm was diluted 10^3 fold with sterile MH broth with ampicillin.
11. 100 µl of the diluted culture was added to entire plate except A12-H12 (negative control)
12. A1 and A11-H11 was the positive control where no antibiotic or small molecule was added. Only culture was added.
13. The plate was read at 600 nm using a plate reader (SpectraMax M2e Plus plate reader).
14. The plate was than incubated at 37°C at static inside a humidity controlled incubator for 12 h.
15. Post read was taken at 600 nm after 12 h of incubation by using the plate reader.
16. Same diluted culture was used to determine the MICs of both antibiotic and small molecule separately in a different 96-well plate to know their individual MICs at same condition.

3.8.3.4.2. Result

Results of checkerboard assay with ciprofloxacin and norfloxacin against *E. coli* KAM32/pUC18-*abeM* is given in Table.3.14 and Table.3.15 respectively. FIC indexes (ΣFIC) for a single concentration (4 µM) of lead molecules are given in the tables. At higher concentrations of small molecules ΣFIC values were much lower. From the checkerboard assay data, it was observed that with increasing concentration of EPI molecules, the MIC of both ciprofloxacin and norfloxacin decreases significantly (Fig.3.10). CCCP, a known efflux pump

inhibitor was taken as a positive control and found similar synergistic relationship with both the antibiotics (Fig.3.11).

Table 3.14 FIC of Ciprofloxacin in combination with lead EPI molecules against *E. coli* KAM32/pUC18-*abeM.*

Lead molecules	MIC of Ciprofloxacin alone (µg/ml), x	MIC of EPI lead molecules (µM), y	MIC in Combination with 4 µM of EPI (µg/ml), z	FIC of Ciprofloxacin A = (z/x)	FIC of lead EPI B= (4/y)	ΣFIC (A+B)
IITR05822	0.05	125	0.013	0.26	0.031	0.291
IITR06474	0.05	250	0.013	0.26	0.016	0.276
IITR08027	0.05	125	0.0031	0.062	0.031	0.093
IITR08678	0.05	250	0.0031	0.062	0.016	0.078
IITR09804	0.05	250	0.0031	0.062	0.016	0.078
IITR09990	0.05	500	0.0063	0.126	0.008	0.134
IITR09990	0.05	250	0.0031	0.062	0.016	0.078
IITR10677	0.05	500	0.0063	0.126	0.008	0.134

Table 3.15 FIC of Norfloxacin in combination with lead EPI molecules against *E. coli* KAM32/pUC18-*abeM.*

Lead molecules	MIC of Norfloxacin alone (µg/ml), x	MIC of EPI lead molecules (µM), y	MIC in Combination with 4 µM of EPI (µg/ml), z	FIC of Norfloxacin A = (z/x)	FIC of lead EPI molecule B= (4/y)	ΣFIC (A+B)
IITR05822	0.31	125	0.039	0.126	0.031	0.157
IITR06474	0.31	250	0.02	0.065	0.016	0.080
IITR08027	0.31	125	0.078	0.252	0.031	0.283
IITR08678	0.31	250	0.078	0.252	0.016	0.267
IITR09804	0.31	250	0.039	0.126	0.016	0.141
IITR09990	0.31	500	0.039	0.126	0.008	0.134
IITR010025	0.31	250	0.02	0.065	0.016	0.080
IITR10677	0.31	500	0.039	0.126	0.008	0.134

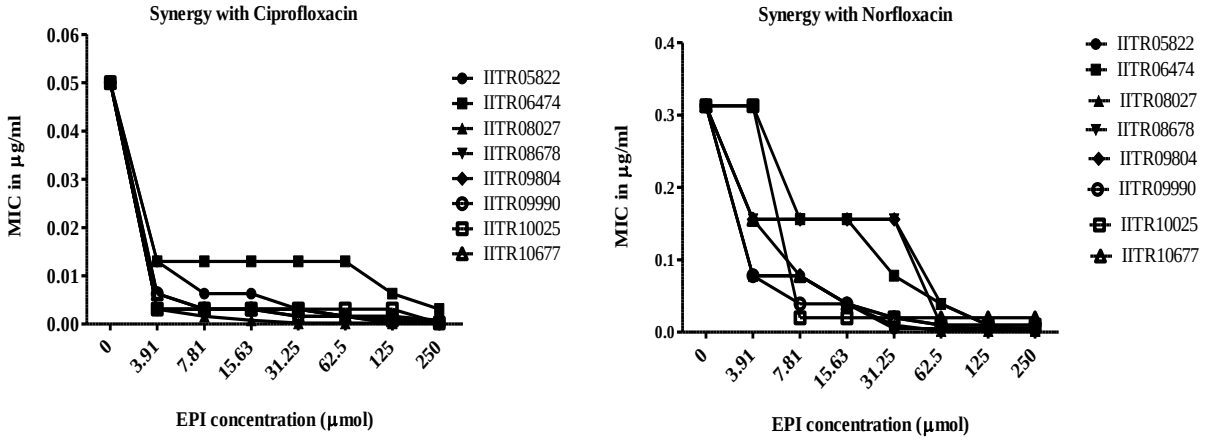


Figure 3.10 Decrease in MIC of Ciprofloxacin and Norfloxacin against *E. coli* KAM32/pUC18-*abeM* in presence of increasing concentration of IITR08027.

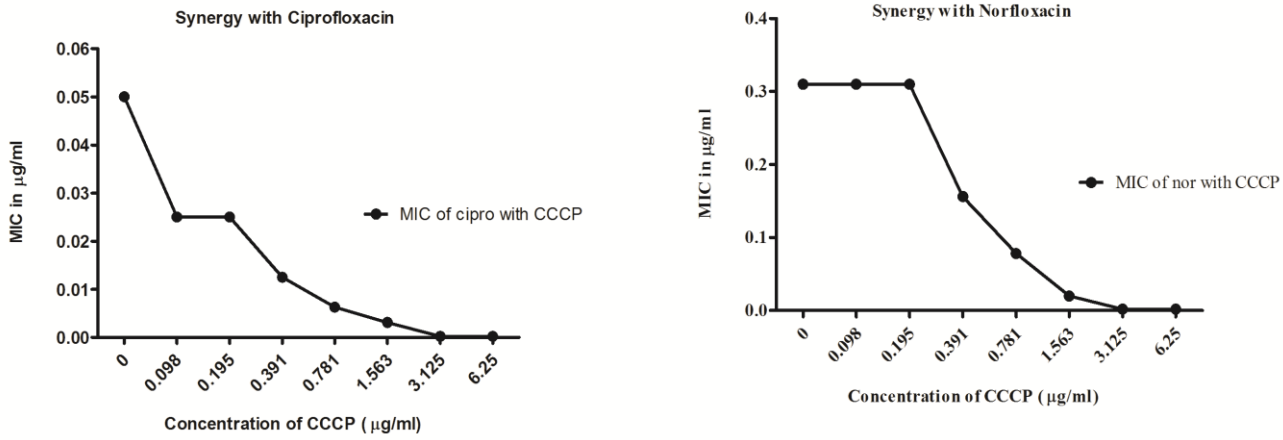
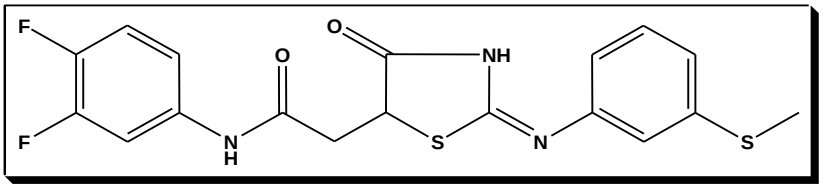


Figure 3.11 Synergistic effect of CCCP (known efflux pump inhibitor) with Ciprofloxacin and Norfloxacin.

3.8.4. Characterization of IITR08027, a potential efflux pump inhibitor of AbeM

IITR08027 appeared to be the most effective molecules out of these 8 potential efflux pump inhibitors (EPIs). Detail characterization of EPI molecules began with the study of IITR08027. The chemical name and structure of IITR08027 is given below (Fig.3.12).



IITR08027

(*E*)-*N*-(3,4-difluorophenyl)-2-(2-(3-(methylthio)phenylimino)-4-oxothiazolidin-5-yl)acetamide

Figure 3.12 Chemical name and structure of IITR08027.

3.8.4.1. EtBr accumulation assay

EtBr is a common substrate for many efflux pumps, including AbeM of *A. baumannii*. The molecule fluoresces when it enters the cells and binds to the cellular components. This fluorescence can be measured and the movement of EtBr from the external environment to the cellular milieu can, therefore, be tracked. Therefore, EtBr accumulation assay was carried out for the characterization of IITR08027 as an efflux pump inhibitor.

3.8.4.1.1. Method

1. The assay was carried out according to the method described previously with minor modifications [83].
2. *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18-*abeM* were grown in LB/ampicillin media until OD_{600} reaches at 0.6.
3. The cells were centrifuged at 10,000 rpm for 1 min and the pellet was washed in equal volume of PBS twice and finally resuspended in PBS, containing 0.4% glucose and adjusted to OD of 0.3 at 600 nm.
4. EtBr was added at 10µg/mL and the fluorescence of EtBr was measured over the time using a SpectraMax M2e Plus plate-reader (Molecular Devices) at excitation and emission wavelengths of 480 nm and 610 nm, respectively for 15 min at an interval of 1 min.
5. Small molecule was added after 15 min at a concentration of 25µM.
6. Fluorescence was measured for next 15 min at an interval of 1 min by using SpectraMax M2e Plus reader (Molecular Devices) at kinetic mode to take readings at time interval of 1 min.
7. The experiment was performed three times and average changes in fluorescence intensity were plotted against time.

3.8.4.1.2. Results

EtBr accumulation assay result suggested that IITR08027 increases the accumulation of EtBr inside the bacterial cells. A sharp elevation in fluorescence intensity was observed when IITR08027 was added to the culture exposed to EtBr (Fig.3.13). It signifies that IITR08027 blocks the AbeM pump and increase the accumulation of EtBr inside the cell.

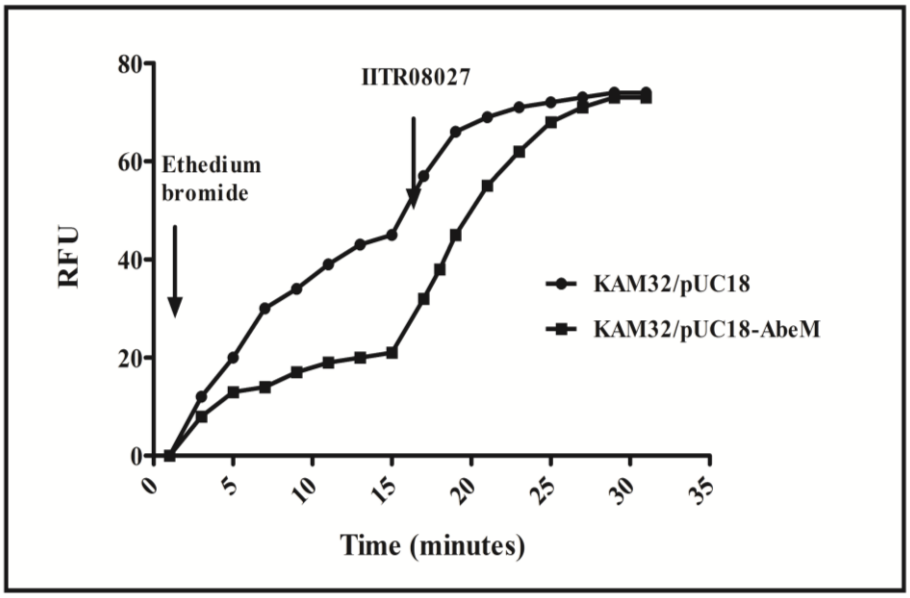


Figure 3.13 Effect of IITR08027 on the accumulation of ethidium bromide (EtBr) in *E. coli* KAM32/pUC18-*abeM* and *E. coli* KAM32/pUC18. Addition of IITR08027 causes a sharp increase in fluorescent intensity indicating that IITR08027 enhances the accumulation of EtBr inside the cells. The accumulation of EtBr in both *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18-AbeM gradually becomes equal owing to inhibition of efflux mechanism by IITR08027. It indicates that IITR08027 inhibit the efflux activity of AbeM leading to the accumulation of EtBr inside the cell.

3.8.4.2. Ciprofloxacin/H^+ antiport assay on everted membrane vesicles of E. coli KAM32/pU18-abeM

AbeM is driven by the proton motive force across the membrane. A constant movement of H^+ and drug (substrate) occurs in opposite direction across the membrane. Efflux of a particular drug molecule (e.g. ciprofloxacin) outside the membrane is proportional to the influx of H^+ in the inner membrane. This antiport movement of H^+ and drug molecules can be monitored though a fluorescence quenching assay of quinacrine, a fluorescent compound. The fluorescence of the molecule gets quenched in presence of H^+ ions. In other words, the fluorescence of quinacrine is inversely related with the H^+ in the system. Once the H^+ is removed from the system, fluorescence of the quinacrine goes up. Therefore, ciprofloxacin/H^+ movement were studied for the further characterization of IITR08027 as an inhibitor of proton motive force.

3.8.4.2.1. Method

3.8.4.2.1.1. Preparation of everted membrane vesicles

1. Everted membrane vesicles was prepared a described earlier with some modification [133,134].

2. *E. coli* KAM32/pUC18-*abeM* Cells were grown in L medium [135] supplemented with 20 mM potassium lactate and 100 μg/ml of ampicillin.
3. The cells were harvested at a late exponential phase of growth, washed with a buffer containing 10 mM Tris-HCl (pH 7.2), 0.14 M KCl, 1 mM 2-mercaptoetanol, and 10% (v/v) glycerol (TKMG buffer) and suspended in same buffer.
4. The cells were broken by a single passage through a French pressure cell using a pressure of 4000 p.s.i. The suspension was centrifuged at 20000 rpm for 30 min for removing the unbroken cells and cell debries.
5. The supernatant was centrifuged at 90000 rpm for 2 h by ultra centrifuge (Beckman Coulter, US). Tubes of 12.5 ml capacity (code.342413, Beckman Coulter) were used.
6. The pellet was washed once with TKMG buffer followed by resuspension in TKMG buffer and total protein was quantified by using BCA protein assay kit (Thermo Scientific, USA).
7. All the steps other than protein estimation were performed at 4°C and the membrane vesicles were kept on ice until use.

3.8.4.2.1.2. Quinacrine fluorescence quenching assay

1. Ciprofloxacin/ H^+ antiport activity was measured by quinacrine fluorescence quenching method with everted membrane vesicle prepared from cells of *E. coli* KAM-32/pUC18-*abeM* as described previously [136].
2. The assay mixture consists of 10 mM Mops-KOH, pH 7.2, 140 mM KCl, 1 μM quinacrine and membrane vesicles (0.2 mg/ml of protein).
3. The fluorescence was measured at excitation and emission wavelength of 420 nm and 500 nm, respectively.
4. 5 mM Lactate was added as an energy source to initiate respiration.
5. As the fluorescence declined and reached at a steady state, ciprofloxacin was added at a concentration of 40 μM. Addition of ciprofloxacin caused a slight increase in fluorescence and again got stabilized.
6. Small molecule (IITR08027) was added at this point at a concentration of 25 μM [137].
7. The experiment was repeated three times and average change in the intensity of fluorescence was plotted against time.

3.8.4.2.2. Results

Addition of lactate to the assay mixture resulted in an influx of H^+ ions owing to respiration and concomitant decrease in the fluorescence of quinacrine (Fig.3.14). Addition of

ciprofloxacin, substrate of AbeM, initiated the drug/H^+ antiport of activity AbeM causing ciprofloxacin to move inside the vesicles in exchange of H^+ ions leading to an increase in quinacrine fluorescence. The antiport was inhibited by addition of IITR08027 which disrupted the proton gradient across the membrane causing an efflux of H^+ ions from the everted membrane vesicles evident by sharp increase in fluorescence of quinacrine. This shows that IITR08027 inhibits the drug/H^+ antiport system by causing the proton gradient across the bacterial membrane to collapse. This energy de-coupling forms the basis of efflux pump inhibition.

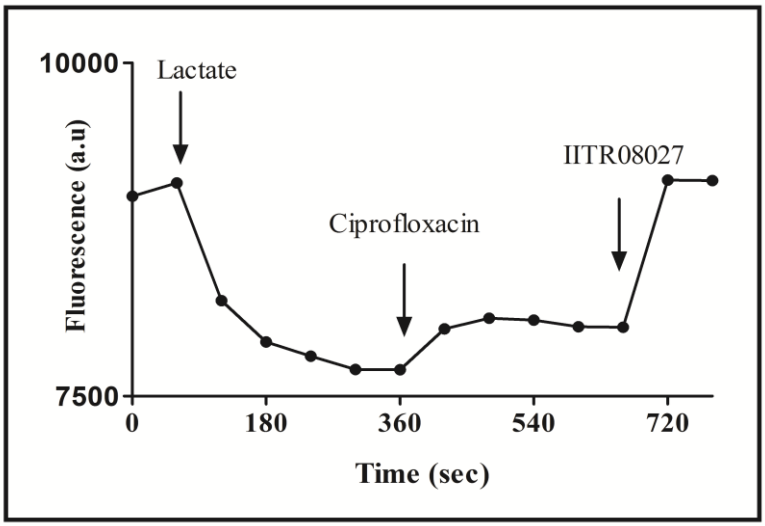


Figure 3.14 Fluorescence quenching to determine inhibition of drug/H^+ antiport: Everted membrane vesicles were prepared from cells of *E. coli* KAM32/pUC18-*abeM*. Potassium lactate (5mM) was added to the reaction mixture to initiate respiration. Movement of H^+ inwards causes a steep decline in fluorescence of quinacrine. After stabilization, ciprofloxacin was added which resulted an increase of fluorescence of quinacrine due to drug/H^+ antiport (protons being pumped out in exchange of drug molecules). On addition of IITR08027 there was a steep increase in fluorescence of quinacrine indicating collapse of the proton gradient and subsequently, inhibition of the drug/H^+ antiport.

3.8.5. Studies with the fluoroquinolone resistant clinical isolate of *Acinetobacter baumannii*

The fundamental objective of this study was to discover an efflux pump inhibitor (EPI) which potentiates the activity of fluoroquinolones against the drug resistant strains of *A. baumannii*. Therefore, for further characterization of IITR08027, multidrug resistant clinical isolates of *A. baumannii* with high resistance for fluoroquinolones have been used in the study.

3.8.5.1. Determination of MIC against fluoroquinolones resistant clinical isolates of A. baumannii in presence and absence of IITR08027

3.8.5.1.1. Method

A number of clinical isolates of *A. baumannii* with high resistance for fluoroquinolones have been tested. As there can be multiple mechanisms for fluoroquinolone resistance, MIC of ciprofloxacin and norfloxacin were determined against all the clinical isolates in presence and absence of IITR08027 similarly as described in section 3.8.3.2.1.

3.8.5.1.2. Result

As shown in Table.3.16 only two of these strains exhibit significant fold of increase in susceptibility toward ciprofloxacin in the presence of IITR08027. RPTC-19 is one of the two clinical isolates which exhibit highest increase in the susceptibility against the ciprofloxacin in the presence of EPI (Table.3.16). On the basis of these observations, RPTC-19, a clinical isolate of *A. baumannii* was chosen for further studies.

Table 3.16 MIC of ciprofloxacin and norfloxacin against the clinical isolates of *A. baumannii* in presence and absence of IITR08027.

Sr. No	Clinical isolates of *A. baumannii*	Without small molecules		With IITR08027		Fold decrease in MIC	
		MIC (µg/ml)		MIC (µg/ml)			
		Ciprofloxacin	Norfloxacin	Ciprofloxacin	Norfloxacin	Ciprofloxacin	Norfloxacin
1	RPTC-02	16	128	16	128	1	1
2	RPTC-04	64	256	4	16	16	16
3	RPTC-07	64	256	32	128	2	2
4	RPTC-08	32	256	32	128	1	2
5	RPTC-12	16	128	16	128	1	1
6	RPTC-13	16	256	16	128	1	2
7	RPTC-16	32	256	16	128	2	2
8	RPTC-17	16	128	16	128	1	1
9	RPTC-19	64	256	1	4	64	64
10	RPTC-22	16	128	16	128	1	1

3.8.5.2. Reverse Transcriptase PCR

Based on the result obtained in section 3.8.5.1, it was hypothesized that expression of AbeM might be the cause of resistance to ciprofloxacin in the strain. The expression of the AbeM pump was confirmed in RPTC-19 by reverse transcriptase PCR.

3.8.5.2.1. Methods

3.8.5.2.1.1. Isolation of total RNA

1. A single colony of *A. baumannii* was inoculated in LB broth and incubated overnight at 37°C with agitation.
2. The overnight culture was diluted to 1% in fresh 5 ml LB broth and incubated at 37°C, with agitation, till OD_{600} = 0.5.
3. 3ml of culture was withdrawn and the cells were harvested by centrifugation at 10000Xg for 1 min at room temperature.
4. The cell pellet, in an RNase free microcentrifuge tube, was resuspended in 1ml of RNA Xpress reagent (HiMedia) and incubated for 10 min at room temperature.
5. 200 µl of chloroform was added to the suspension, mixed vigorously and again incubated for 10 min at room temperature.
6. The microcentrifuge tube was centrifuged at 12000Xg for 15 min at 4ºC.
7. The supernatant was collected carefully avoiding the protein interface between aqueous and organic layers.
8. The supernatant was transferred into a fresh RNase free microcentrifuge tube and 500 µl of isopropanol was added. The RNA was allowed to precipitate at room temperature for 15 min.
9. The microcentrifuge tube was centrifuged at 12000Xg for 12 min and the supernatant was carefully decanted.
10. The pellet was washed with 75% Ethanol (prepared in DEPC treated water) and centrifuged at 7500Xg for 5 min.
11. The supernatant was discarded and the RNA pellet was allowed to air dry at room temperature.
12. Upon drying the pellet was resuspended in 30 µl of DEPC treated water and incubated at 55°C for 10 min with gentle intermittent vortexing.
13. The dissolved RNA was run on 2% agarose gel to check its consistency.

3.8.5.2.1.2. DNase Treatment to the RNA sample

DNase treatment of RNA samples was done using Fermentas DNase I enzyme. The following reaction mixture was prepared.

Ingredient	Volume (µl)
RNA sample	25.0
10X DNase buffer with $MgCl_2$	3.5
DNase I	2.0
DEPC treated water	4.5
Total	**35.0**

The mixture was incubated at 37ºC for 20 min. DNase I was inactivated by adding 3.5µl of 25mM EDTA solution and subsequent 10 min incubation at 70ºC. DNase treated RNA was again run on 2% agarose gel to check.

3.8.5.2.1.3. Synthesis of cDNA

First strand DNA synthesis from RNA sample was carried out using SuperScript III RT (Life technologies). Following reaction mixture was prepared

Ingredient	Volume (µl) or amount
RNA sample (164 ng/µl)	3.0
10uM Random hexamer primer	4.0
10mM dNTP	5.0
Water	1.0
Total	**13 µl**

The mixture was mixed well and heated at 65ºC for 5 min. Following were added to the reaction mix

Ingredient	Volume (µl) or amount
5X FS synthesis buffer	4.0
0.1M DTT	1.0
RNase inhibitor	1.0
SuperScript III RT	1.0

Samples were incubated at 25ºC followed by 1 h incubation at 55ºC. The enzyme was inactivated by 15 min incubation at 70ºC. cDNA was quantified by using a Nanodrop (Thermo Scientific).

3.8.5.2.1.4. PCR reaction mixture and PCR program

Reagents	Stock concentration	Final concentration	Volume (µl) used in 50 µl reaction
Primer Forward	10 µM	0.4 µM	2
Primer Reverse	10 µM	0.4 µM	2
Pfu Buffer	10X	1X	5
dNTPs	2 mM	0.2 mM	5
Pfu Polymerase enzyme	2.5 U/µl	2.5 U	1
Template cDNA	100 ng/µl	100 ng	1
Nuclease free water	-	-	34
		Total	**50 µl**

3.8.5.1.1.5 PCR Program used for the amplification of internal region of abeM

Steps	Temperature	Duration	Cycles
1	98°C	2 min	
3	98°C	30 s	30 cycles
	52°C	30 s	
	72°C	45 min	
4	72°C	10 min	
5	4°C	Hold	

3.8.5.2.2. Result

Amplification of a coding segment of AbeM was observed after reverse transcriptase PCR (Fig.3.15). This observation confirmed the expression of *abeM* in the *A. baumannii* RPTC-19. On the basis of this observation RPTC-19 has been used in the study for further characterization of IITR08027.

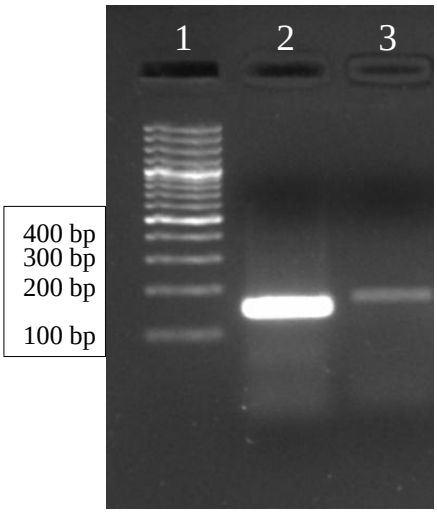


Figure 3.15 Expression of *abeM* in *A. baumannii* RPTC-19, a clinical isolate of *Acinetobacter baumannii* resistant to fluoroquinolones. Total RNA was isolated from *A. baumannii* RPTC-19 cells at exponential growth stage. Reverse transcriptase PCR (RT-PCR) for 16SrRNA (lane 2) and *abeM* (lane 3) was performed using the total RNA, after revere transcription, using primers listed in Table.3.2. Lane 1 contains 100bp DNA ladder.

3.8.5.3. Determination of Minimum Effective Concentration (MEC) of ciprofloxacin and norfloxacin against the clinical isolate of A. baumannii, RPTC-19

MEC (Minimum Effective Concentration) is defined as minimal concentration of EPI that produced the maximal reduction in MIC of an antibiotic. MECs of ciprofloxacin and norfloxacin in combination with IITR08027 were determined against the clinical isolate RPTC-19 as described previously [104].

3.8.5.3.1. **Method**

1. Assay was done in 96 well plate using Muller Hinton broth by using two fold broth dilution methods as described in section 2.13.1.
2. MICs of both ciprofloxacin and norfloxacin were determined in presence of increasing concentration of IITR08027.
3. The antibiotics were tested at 10 different concentrations, while small molecule was tested at 8 different concentrations.
4. Inhibitory concentration of antibiotics (ciprofloxacin and norfloxacin) was plotted against the increasing concentration of small molecule.

3.8.5.3.2. **Result**

A remarkable decrease in MICs of both ciprofloxacin and norfloxacin against RPTC-19 were observed with increasing concentration of IITR08027 (Fig.3.16). The MEC (Minimum

Effective Concentration) of IITR08027 for ciprofloxacin and norfloxacin were observed 16 μM and 32 μM respectively. Alone the MICs of ciprofloxacin and norfloxacin against the clinical isolate were 64 μg/ml and 256 μg/ml respectively. Therefore, combination therapy of IITR08027 with ciprofloxacin and norfloxacin can be extremely effective against quinolone resistant clinical isolates of *A. baumannii* expressing AbeM or its homologue.

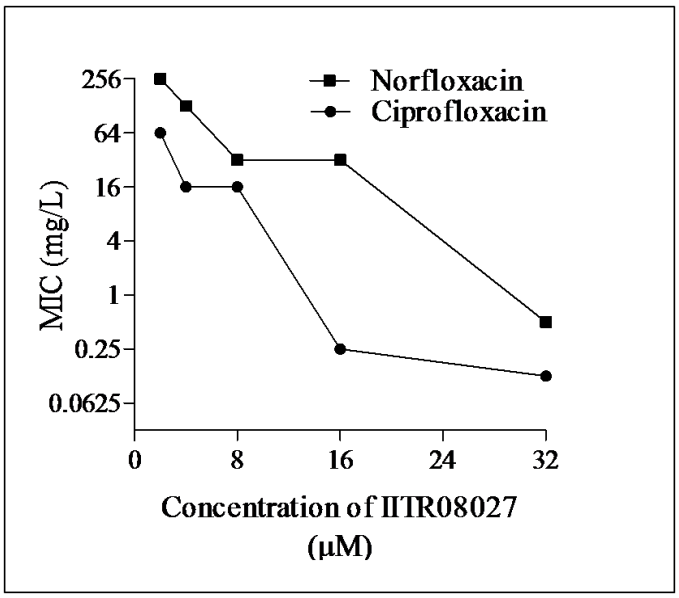


Figure 3.16 Determination Minimum Effective Concentration (MEC) of ciprofloxacin and norfloxacin in combination with IITR08027: Efficacy of ciprofloxacin and norfloxacin in combination with IITR08027 was determined against the clinical isolate of *Acinetobacter baumannii* RPTC-19. Minimum effective concentration (MEC) of IITR08027 for ciprofloxacin and norfloxacin against *Acinetobacter baumannii* RPTC-19 was determined as 16 μM and 32 μM respectively.

3.8.5.4. Kill kinetic Assay

Efflux pump inhibitor may also influence the killing kinetics of an antibiotic [104,119]. Therefore, time kill assay of ciprofloxacin alone and in combination with IITR08027 was carried out against the clinical isolate of *A. baumannii*, RPTC-19.

3.8.5.4.1. Method

1. Kill kinetics of ciprofloxacin in the presence of IITR08027 was evaluated by a time-kill method as described previously [104] .
2. A single colony of *Acinetobacter baumannii* clinical isolate (RPTC-19) was inoculated in MH broth and incubated overnight at 37°C with agitation.
3. The overnight culture was diluted 100 times in fresh MH medium.

4. 5 ml of diluted inoculums was treated with 64 µg/ml ciprofloxacin alone (1X MIC), 16 µg/ml ciprofloxacin alone (1/4 X MIC) and 16 µg/ml ciprofloxacin in combination with 50 µM IITR08027 (1/4 X MIC + EPI).
5. One tube, 5 ml culture was kept as a control without any treatment with antibiotics and incubated in same condition at 37°C for 12 h.
6. 100 µl of samples were withdrawn after 3 h interval and plated onto freshly prepared MH agar plates with different dilutions. Fresh MH media was used for serial dilution.
7. The plates were incubated at 37°C overnight. The colonies appeared on MH agar plates were counted and total CFU/ml was calculated by multiplying with the dilution factor.
8. Change in $\log_{10}$CFU/ml of bacterial cells was plotted against time.

3.8.5.4.2. Result

It was observed that IITR08028 enhances the killing efficiency of ciprofloxacin in presence of EPI. The time kill curve of *Acinetobacter baumannii* clinical isolate (RPTC-19) with ciprofloxacin at sub-inhibitory concentration (1/4 MIC) in presence and absence of IITR08027 were performed (Fig.3.17). Sub-inhibitory concentration of ciprofloxacin 16 µg/ml (1/4 MIC) alone could not inhibit the growth of the organism. However, sub-inhibitory concentration of ciprofloxacin exhibited bactericidal activity in presence of 25 µM of IITR08027.

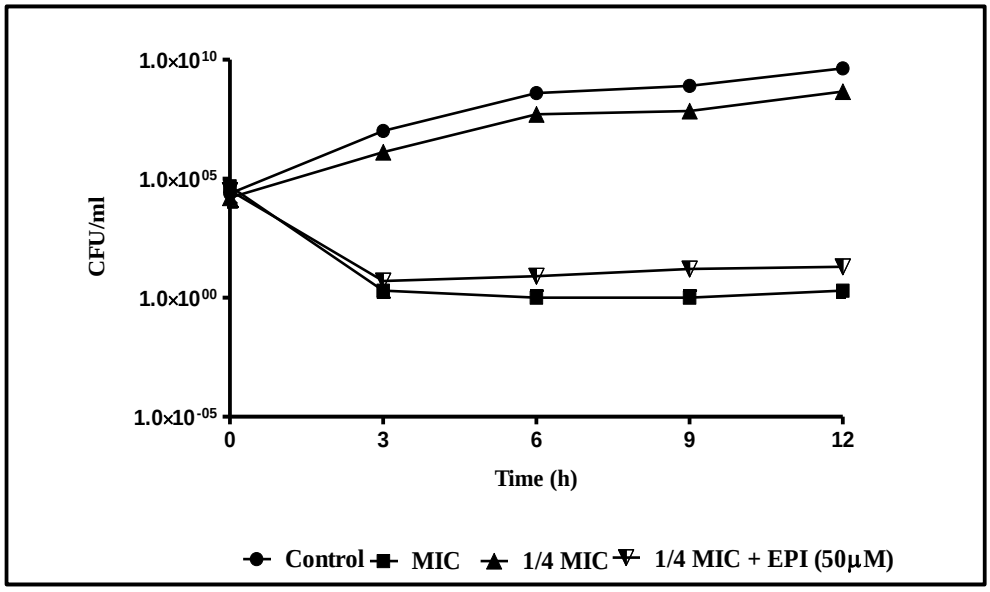


Figure 3.17 Time kill kinetics of ciprofloxacin in presence and absence of IITR08027: Sub-inhibitory concentration of ciprofloxacin was found to exhibit bactericidal activity against the clinical isolate, *A. baumannii* RPTC-19 in combination with IITR08027.

3.8.5.5. In vitro post-antibiotic effect

Post Antibiotic Effect (PAE), delayed regrowth of bacteria following exposure to an antibiotic is an pharmacodynamic characteristics of an antibiotic [100]. It is an important parameter because it is crucial for determining the correct dosing of antibiotic [101,138]. Longer delay in growth is always desirable for a good antibiotic after a brief exposure [139]. It also gives an idea how persistent an antibiotic is or how long the bacteria can sustain the action of antibiotics. Therefore, the influence of IITR08027 on the PAE of ciprofloxacin was examined against the clinical isolate, RPTC-19.

3.8.5.5.1. Method

1. *In vitro* post-antibiotic effect of ciprofloxacin on clinical isolate of *A. baumannii* (RPTC 19) in presence and absence of IITR08027 was determined as described previously with some minor modifications [101].
2. 5 ml of overnight culture (OD was adjusted to 0.1 by dilution with fresh media) in Muller Hinton broth was incubated for 2 h at 37°C with and without ciprofloxacin at concentration of 10 X MIC (640 μg/ml).
3. In another set of tubes, ciprofloxacin at 10X MIC along with increasing concentrations (50 μM, 100 μM and 150 μM) of IITR08027 were also incubated at same condition.
4. After 2 h of incubation, antibiotic was removed by centrifugation of the culture at 7000 rpm for 10 min.
5. The cells were washed twice with the phosphate buffer and pellets were resuspended in fresh MH broth.
6. The OD of the tubes without the antibiotic was diluted by adding fresh media. OD of culture at 600 nm in all the culture tubes were made equal to 0.06.
7. The initial OD at 600 nm of all the tubes was 0.06.
8. 200 μl of culture was withdrawn after every 1 h and OD was measured by SpectraMax M2e Plus spectrophotometer (Molecular Devices).
9. Simultaneously, 50μl of samples were plated on freshly prepared MH agar plate with different dilutions.
10. Colonies appeared on MH agar plates after overnight incubation were counted and CFU/ml was calculated. A linear regression line was fitted to the $\log_{10}$ (CFU/ml) values over the period of 0 to 4 h after the exposure to the antibiotic as described previously [140].
11. The PAE was determined according to (Craig *et al*, 1999) as PAE = T – C, where T is the time required for the visible counts of the exposed bacteria to increase by 1 $\log_{10}$ above the

counts observed immediately after washing and C is the corresponding time for the antibiotic unexposed control.

***The period of zero to 4 h was chosen for comparison considering the following facts: it was the period immediate after the exposure of antibiotics, it was long enough duration for studying such a fast growing resistant mutant and visible growth was observed after 4 h.

3.8.5.5.2. Result

PAE of ciprofloxacin on the *A. baumannii* RPTC19 was found to be extended in presence of IITR08027 which is a potential efflux pump inhibitor (EPI). With increasing concentration of IITR08027 (EPI) recovery time for bacterial culture was lingered. In other words, bacterial cells took more time to come back at its normal growth phase when it was treated together with ciprofloxacin and IITR08027 as compared to ciprofloxacin alone (Fig.3.18 and 3.19). The PAE of ciprofloxacin alone and in combination with IITR08027 was determined (Table.3.17).

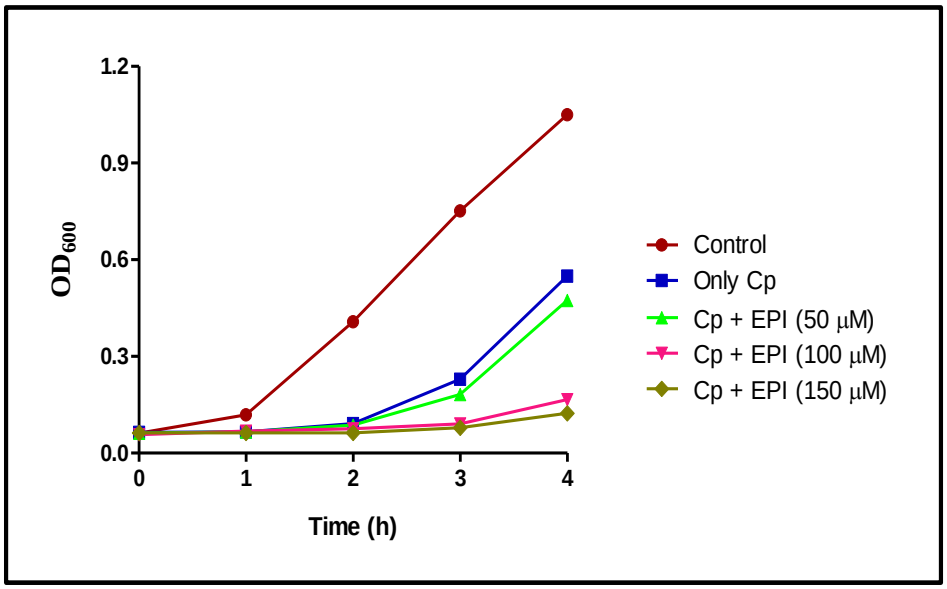


Figure 3.18 Induction of Post antibacterial effect (PAE) by ciprofloxacin alone and in combination with IITR08027 against the clinical isolate *A. baumannii* RPTC-19: *A. baumannii* RPTC-19 cells at OD_{600} of 0.1 were treated with 10X MIC of ciprofloxacin alone (Cp alone) and same concentration of ciprofloxacin in combination with 50 µM, 100 µM and 150 µM of IITR08027 (Cp+EPI) for 2 h. The antibiotic was removed after 2 h and the cells were suspended in fresh media. Initial OD_{600} of the cells, in different conditions, was made equal by adding sterile media and growth was monitored for 4 h with an interval of 1 h. Each point is representative of mean of triplicate values and error bars represent standard deviation.

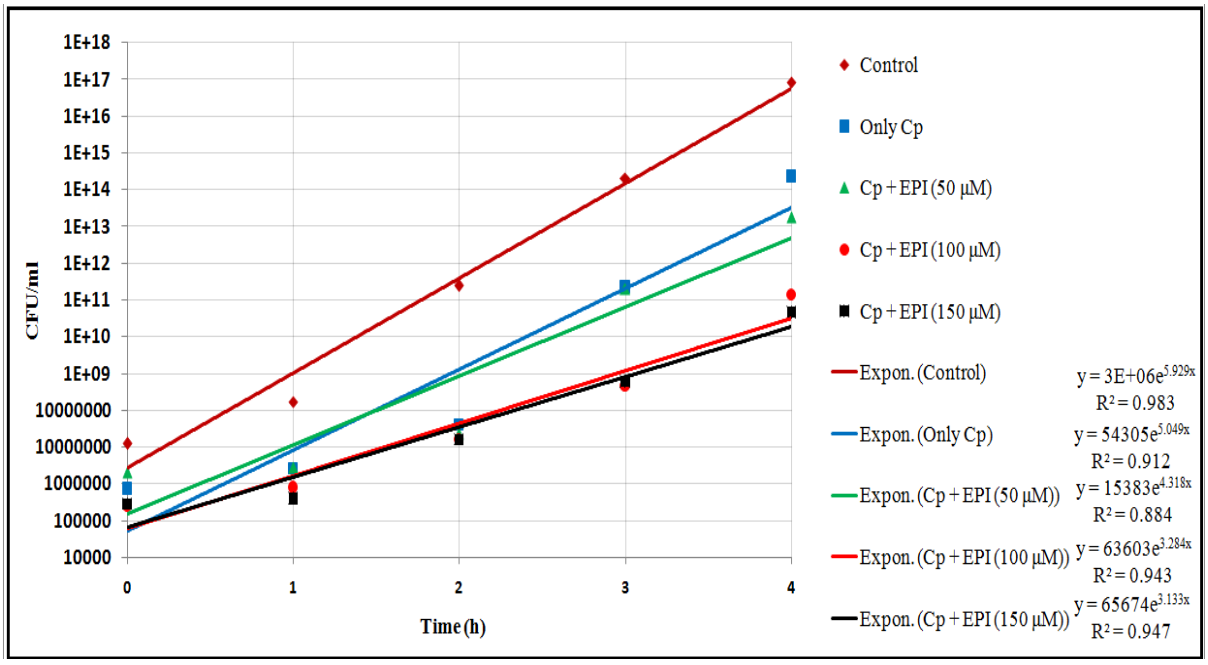


Figure 3.19 Induction of Post antibacterial effect (PAE) by ciprofloxacin alone and in combination with IITR08027 against the clinical isolate of *A. baumannii* (RPTC-19): A log phase culture of 0.1 OD cells was treated with 10 X MIC of ciprofloxacin alone and same concentration of ciprofloxacin with increasing concentration of IITR08027 for 2 h. The antibiotics were removed after 2 h and the cells were suspended in fresh media. Initial OD of the cells was made equal by adding sterile media. The culture was incubated in shaking condition at 37°C for 4 h. 50µl of samples were withdrawn at every h and plated on freshly prepared MH agar plate with different dilutions to determine the viable counts (CFU/ml). The PAE, average time required for increase in unit logarithmic scale was determined (Table.3.17).

Table 3.17 Post antibiotic effect (PAE) of ciprofloxacin on *Acinetobacter baumannii* RPTC-19 in the presence and absence of IITR08027.

	$\log_{10}$ CFU/ml/h	**Time required for unit $\log_{10}$ increase (min)**	**PAE (min)**
Control	2.75	22	-
Only Ciprofloxacin	2.05	29	7
Ciprofloxacin + IITR08027 (50 µM)	1.75	34	12
Ciprofloxacin + IITR08027 (100 µM)	1.325	45	23
Ciprofloxacin + IITR08027 (150 µM)	1.25	48	26

3.8.6. Selection of resistant mutant *in vitro*

Another very important characteristic of an efflux pump inhibitor is its ability to reduce the frequency of resistant mutant development [104,119]. A mutant selection study was carried

out on *A. baumannii* ATCC1798 in presence and absence of IITR08027 with varying concentration of ciprofloxacin.

3.8.6.1. Method

1. Emergence of ciprofloxacin resistant mutant in presence and absence of IITR08027 was monitored as described previously [104].
2. *Acinetobacter baumannii* (ATCC 17978) culture of approximately 10^9 CFU/100 µl was plated on MH agar plate containing ciprofloxacin at a concentration of 2, 4, 8 and 16 times of its MIC (µg/ml).
3. The same concentrations of ciprofloxacin were also tested in combination with IITR08027 at a concentration of 50 µM.
4. Frequency of ciprofloxacin resistant mutant was determined as described previously [141] by dividing the total number of colonies appeared after 48 h of incubation at 37°C on the drug containing plate by the total number of CFU plated.

3.8.6.2. Results

Mutant selection frequency was decreased in presence of IITR08027. It was observed that same concentration of ciprofloxacin produces lesser number of mutants when treated with IITR08027. No mutant was selected at 8X MIC concentrations which can be defined as mutant prevention concentration (MPC) of ciprofloxacin against RPTC-19. At 2X and 4X MIC concentrations, the frequency of resistant mutant was much higher in comparison to same concentration with IITR08027 (Table.3.18).

Table 3.18 Frequency of selection of ciprofloxacin resistant mutants in *Acinetobacter baumannii* ATCC 17978 in the presence and absence of IITR08027.

	Mutant selection frequency		
	2 X MIC (128 mg/L)	**4 X MIC (256 mg/L)**	**8 X MIC (512 mg/L)**
Without IITR08027	3×10^{-7}	1.85×10^{-7}	$< 10^{-9}$
With IITR08027 (50 µM)	1×10^{-7}	6×10^{-9}	$<10^{-9}$

3.8.7. MTT assay

In order to assess the cytotoxicity of IITR08027 was determined by MTT assay on mammalian Hela cells.

3.8.7.1. Method

1. Cytotoxicity of IITR08027 on Hela cells was determined by the MTT assay as described previously [142].
2. In brief, approximately 5×10^3 cells in 200 µl of media were seeded in a 96-well microtiter plate and incubated overnight.
3. Cells were then treated with increasing concentrations of IITR08027 (1 to 200 µM) and incubated at 37°C for 24 h.
4. Control cells were treated with 0.1% DMSO (vehicle control) and incubated at same condition for 24 h.
5. After 24 h, 20 µl MTT (5 mg/ml) were added to the culture and incubated for 4 h at 37°C.
6. MTT-containing medium was then removed very carefully and 200 µl DMSO was added to dissolve the formazone crystal by gentle agitation.
7. Optical density (OD) was measured at 570 nm in a plate reader (M2e, Molecular Devices). Absorbance values were expressed as percentage of solvent control.
8. Percentage inhibition was calculated in comparison to the solvent control. The percentage inhibition was calculated as

   % inhibition = 100 − [(Mean OD of treated cell×100)/mean OD of vehicle treated cells]
9. The IC50 values were calculated using graph pad prism, version 5.02 software (Graph Pad Software Inc., CA, USA).
10. Each assay was done three times, and the results of one representative experiment are shown in

3.8.7.2. Result

The MTT toxicity study against mammalian Hela cells showed that IITR08027 has minimum toxicity at its effective concentration range. The minimum effective concentration (MEC) of IITR08027 was found to be around 16 µM against highly resistant clinical isolate of *Acinetobacter baumannii* (RPTC-19). As shown in Fig.3.20 at 80 µM (5X MEC) concentrations, cell viability of Hela cell line was around 80 percent. However, at higher concentration (more than 100 µM) the molecule exhibited some level of toxicity. The IC50 of the molecule is around 150 µM (133.33 ± 15.4 µM).

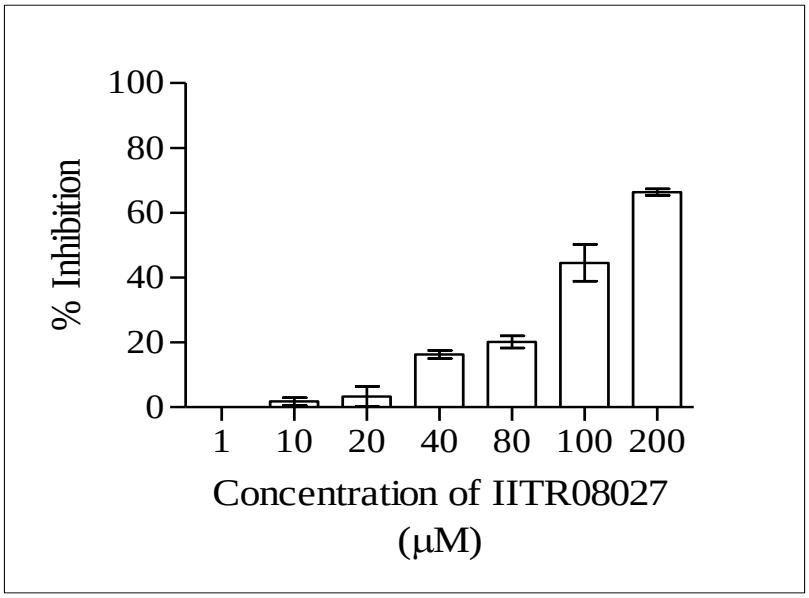


Figure 3.20 Cytotoxicity of IITR08027: Cytotoxicity of IITR08027 was determined by MTT assay. Percentage inhibition of cell growth was plotted against the concentration of IITR08027 used for cell treatment.

This study has demonstrated a viable screening strategy for identifying an efflux pump inhibitor against a specific transporter. IITR08027 has been identified and characterized as an efflux pump inhibitor of AbeM in *A. baumannii*. AbeM is a clinically relevant efflux pump and no specific inhibitors have yet been reported against this pump. IITR08027 was found to potentiate the activity of fluoroquinolones as well as many other substrate antibacterials against multidrug resistant mutant of *A. baumannii*. The molecule also improves the pharmacodynamic properties of quinolones as an antibiotic by enhancing the killing potential and lingering the PAE. The molecule itself is noninhibitory upto a very high concentration and very less toxic at its effective range. The lead molecule can further be taken up for preclinical trials and animal toxicity studies.

CHAPTER 4

DISCUSSION AND CONCLUSION

CHAPTER 4
DISCUSSION AND CONCLUSION

4.1 Discussion

Encountering multiple drug resistance in bacterial pathogens is a serious challenge for healthcare. New antibiotics are not available since long time and old antibiotics are becoming ineffective against bacteria causing a number of infectious diseases in human and other animals. Discovering new antibacterial molecules and rejuvenating the old antibiotics are the need of this h to tackle the situation [143]. This study centres around both of these two aspects of combating the multiple drug resistance in bacterial pathogens. Small molecules with diversified chemical structures have been screened by cell based assay in both the approaches for discovering novel chemical entities.

4.1.1 Identification and characterization of novel antibacterial molecules

The first approach of the study was to discover novel chemical entities against gram negative bacteria. Here is the summary of the outcome of the initial study.

4.1.1.1. Screening of a small molecule library consists of 10956 molecules

The study has demonstrated a systematic approach of screening a large collection of small molecules within a standard (common) laboratory facility without any intervention of robotic system. All 10956 molecules were tested individually against *E. coli* ATCC25922 for discovering novel antibacterial small molecule (Fig.2.18). This study can be adapted for screening against any other bacteria with different collection of molecule from natural as well as synthetic sources even in an academic setup [27].

4.1.1.2 Discovery of a molecule with broad spectrum antibacterial activity

The study led to the identification of IITR06144 (Fig.2.20), a novel antibacterial small molecule with broad spectrum antibacterial activity (Table.2.2). The molecule inhibits the growth of number of multi drug resistant pathogens including *Acinetobacter baumannii* AYE, *E. coli* O157 and *Vibrio fluvialis* BD146 [144].

4.1.1.3. Low toxicity of IITR06144

Toxicity of IITR06144 was determined by Hemolysis assay with human red blood cells and MTT assay with C3H10T1/2 stem cells (Table. 2.3 and 2.4). The toxicity of IITR06144

was compared with two commercially available nitro aromatic drugs namely nitrofurantoin and furazolidone. The relative toxicity of the molecule was lower than those two nitroaromatic drugs.

4.1.1.4. IITR06144 interfere with bacterial cell division

Cell division proteins are attractive drug targets for future antibiotics [145]. Resistance is developing against the drugs with conventional common targets. In that context, it is important to identify molecule with novel therapeutic target. IITR06144 restricts bacterial cell division process. It prevents two daughter cells to be separated from each other during cell division leading to cellular elongation. Microscopic evidences which support this hypothesis are given in Fig.2.22-2.24.

4.1.1.5. IITR06144 inhibit FtsZ by delocalization of FtsZ

FtsZ is reported as a target of many antibacterial agents. Filamentation or cellular elongation are common phenomenon associated the mechanism of all the FtsZ inhibitors [146-153]. Exposure of IITR06144 leading to cellular filamentation in *E. coli* is evident in section 4.1.1.3. It is also evident from the experimental data (given in section 2.17.2) that IITR06144 causes delocalization of FtsZ in the entire cell (Fig.2.6). A uniform distribution of FtsZ-GFP was observed in FtsZ over expressor of *E. coli* cells (Aska clone). But, once it was treated with IITR06144 at sub-inhibitory concentration, the FtsZ was found to be delocalized into the entire cells. Delocalization of FtsZ assembly was evident by the dissolution of GFP in the entire cells. The dissolution of GFP was visualized by fluorescence microscopy (Fig.2.6).This is direct evidence that demonstrates that IITR06144 inhibits FtsZ leading to delocalization of FtsZ in entire cell. Because of FtsZ delocalization, division ring (septum) cannot be formed in mid-cell region. As consequence, two daughter cells cannot be separated from each other leading to cellular filamentation.

4.1.1.6. Genetic evidence of FtsZ inhibition by IITR06144

Genetic evidence of inhibiting FtsZ by berberine was proved earlier by antisense RNA mediated gene silencing study [45]. A similar study, given in section 2.17.3 has revealed that IITR06144 inhibit FtsZ. The growth kinetic data (Fig.2.28) indicates that increase in expression of *ftsZ* antisense RNA causes a simultaneous increase in the activity (potency) of IITR06144. It suggests that both the antisense RNA of *ftsZ* and IITR06144 inhibit the same target FtsZ.

4.1.1.7. The molecule does not function through slmA and minC

One assumption was that IITR06144 might inhibit FtsZ in an indirect manner, through other internal players (other proteins that inhibit FtsZ polymerisation). SlmA, nucleoid occlusion factor puts a hold in bacterial cell division until and unless chromosomal DNA has been segregated properly in two daughter cells. It does not allow two daughter cells to be separated by inhibiting FtsZ until the DNA replicates properly and segregated [57]. Another inhibitor of FtsZ is MinC which inhibits FtsZ at polar region [154]. MIC data (Fig.2.29) with the knockout mutants (Keo collection) of *slmA* and *minC* does not show any significant change in the susceptibility of these mutants against IITR06144.It suggests that inhibition of FtsZ by IITR06144 is not mediated via the action of either SlmA and MinC.

4.1.1.8. Major nitrofuran reductase NfsA augments the activity of these molecules

NfsA and NfsB are the major nitro reductases found in *E. coli* responsible for reducing nitro group (-NO_2) of the nitroaromatic compounds [66]. MIC data with *nfsA* knockout (Keo collection) and over-expression mutant (Aska library) of *E. coli* suggest that NfsA influence the activity (potency) of IITR06144 inside the bacterial cells. It was observed that the MIC of IITR06144 against *ΔnfsaA* of *E. coli* is twofold higher than the wild type stain (Fig.2.29). It suggests that in absence of NfsA, potency of IITR06144 decreases. Somehow the activity of IITR06144 is elevated by NfsA function. This hypothesis was reconfirmed by a similar kind of experiment. In this case, MIC was determined against the overexpressor of NfsA in inducing condition (section 2.17.5). A remarkable increase the sensitivity of *E. coli* overexpressor was observed against IITR06144 (Fig.2.30). From both of these observation, it is hypothesized that NfsA might reduce the –NO_2 group and convert it to a more reactive –NH in a stepwise manner as described previously [61,155].

4.1.2 Identification and characterization of novel efflux pump inhibitor

The second approach of the study was to discover an efflux pump against, AbeM of *A. baumannii* and reviving the activity of fluoroquinolones against quinolone resistant *A. baumannii*. Here is the summary of the outcome of second approach.

4.1.2.1 Screening of 8000 small molecules for potential Efflux Pump Inhibitors (EPIs)

A systematic approach of screening a small molecule library of 8000 molecules has been demonstrated. All 8000 molecules were tested individually against *E. coli* KAM32/pU18-*abeM* in 96-well plates (Fig.3.6). This approach is also applicable for discovering efflux pump

inhibitor against other efflux pumps similar to AbeM in any other organism. A simple and cost effective primary screening strategy was developed for screening. The relative resistance of *E. coli* KAM32 cells harboring multiple copies of *abeM* cloned into a multi copy number plasmid has been utilized for screening.

4.1.2.2 Identification of novel efflux pump inhibitor, IITR08027

Study led to the identification of IITR08027 (Fig.3.12), an efflux pump inhibitor of AbeM in *Acinetobacter baumannii*. The molecule has been characterised as an efflux pump inhibitor of AbeM by EtBr accumulation assay (section 3.8.4.1) and drug/H^+ antiport study (section 3.8.4.2). The utility of the molecule for using in combination with fluoroquinolones (ciprofloxacin and noxfloxacin) were also studied in detail (section 3.8.5.3-5)

4.1.2.3 IITR08027 potentiates the activity of multiple substrates of AbeM

IITR08027 was found to potentiate the activity of a number of antibiotics, especially the fluoroquinolones, against *E. coli* KAM32/pUC18-*abeM* (Table.3.13). 16 to 32 fold decrease in MIC of ciprofloxacin and norfloxacin was observed in presence of 50 μM of IITR08027. Apart from fluoroquinolones, 4 fold decrease in the MIC of kanamycin and 8 fold of decrease in MIC of ofloxacin and chlorohexidine was also observed. Addition of IITR08027 also resulted in significant fold decrease in MIC of other substrates like EtBr, DAPI, Hoechst 33342 and acriflavin.

4.1.2.4 IITR08027 enhances the accumulation of EtBr inside the cells

EtBr is a common substrate for efflux pumps. Fluorescence intensity goes up as EtBr accumulates inside the cell. Therefore, accumulation of EtBr inside the cells can be measured through changes in fluorescence intensity. EtBr accumulation assay was carried out to demonstrate whether IITR08027 facilitates the accumulation of EtBr inside the bacterial cells (section 3.8.4.1). The result indicated that IITR08027 enhance the accumulation of EtBr inside the bacterial cells (Fig.3.13). It suggests that IITR08027 inhibits the efflux activity of AbeM leading to the accumulation of EtBr inside the cells. These finding qualify IITR06144 as an efflux pump inhibitor of AbeM.

4.1.2.5 IITR08027 restricts the antiport movement of ciprofloxacin/H^+ across the membrane

Most of the secondary transporters including AbeM are driven by the electrochemical gradient across the membrane. They are primarily antiport system where cations (e.g. H^+ and Na^+) and the drugs (e.g. ciprofloxacin) move opposite to each other. It is an essential and

intrinsic quality of a secondary pump. AbeM is driven by the H^+ gradient across the membrane. It is an antiport system where H^+ and the fluoroquinolones (substrate) moves opposite to each other. Qunacrine fluorescence quenching assay was carried out to determine whether IITR08027 inhibit this antiport movement (section 3.4.8.2). The result of the study (Fig.3.14) suggested that IITR08027 inhibit the antiport movement of ciprofloxacin/H^+ across the bacterial membrane.

4.1.2.6 IITR08027 enhances the efficiency of fluoroquinolones against multidrug resistant A. baumannii

Not only in *E. coli* KAM32/pUC18-*abeM*, IITR08027 enhances the efficiency of both ciprofloxacin and norfloxacin against multi drug resistant clinical isolates of *A. baumannii* expressing AbeM or its homologue. MICs of both ciprofloxacin and norfloxacin with varying combinations of IITR08027 were determined against fluoroquinolone resistant clinical isolate of *A. baumannii* (section 3.8.5.3.) The result suggests that MICs of both the antibiotics decrease with gradual increase in concentration of IITR08027 (Fig.3.16). The minimum effective concentration (MEC) of ciprofloxacin and norfloxacin against the clinical isolate of *A. baumannii* were determined as 16 μM and 32 μM respectively.

4.1.2.7 IITR08027 enhances the killing efficiency of ciprofloxacin

Kill kinetics of ciprofloxacin and varying concentrations of IITR08027 was determined (section 3.8.5.4.). From the result (Fig.3.17), it was observed that killing potential of ciprofloxacin was augmented in combination with IITR08027. Sub-inhibitory concentration of ciprofloxacin at 16 μg/mL (1/4 MIC) alone could not inhibit the growth of the organism. However, sub-inhibitory concentration of ciprofloxacin along with IITR08027 at a concentration of 50 μM restricted the bacterial growth up to 12 h.

4.1.2.8 IITR08027 extends the post antibiotic effect (PAE) of ciprofloxacin

Post antibiotic effect (PAE) is the amount of time required for one log increase in cell population after the antibiotic has been withdrawn. Post antibiotic effect of ciprofloxacin alone and in combination with IITR08027 was determined (section 3.8.5.5). The result (Table.3.17) suggested that in presence of IITR08027 the PAE of ciprofloxacin was extended. The time for recovery in growth was higher in combination with IITR08027 (Fig. 3.18 and 3.19).

4.1.2.9 IITR08027 reduces the frequency of resistant mutant selection

Mutant selection frequency was found to be decreased in presence of IITR08027 (Table.3.18). It was observed that same concentration of ciprofloxacin produces lesser number of mutants when treated in combination with IITR08027.

4.1.2.10 IITR08027 is nontoxic in its effective rage

The minimum effective concentration (MEC) of IITR08027 was observed 16 μM and 32 μM for ciprofloxacin and norfloxacin against highly resistant clinical isolate of *Acinetobacter baumannii*. The MTT toxicity study against mammalian HeLa cells showed that IITR08027 has very low cytotoxicity at this effective concentration range (Fig.3.20).

4.2 Conclusion

The entire study was focused on identification and characterization of novel chemical entities against multiple drug resistant gram negative bacterial pathogens. Diverse collection of small molecules have been used effectively developing simple phenotype based screening strategies for identifying novel antibacterials as well as efflux pump inhibitors (EPIs). The study can be adopted for targeting any other pathogens or other families of efflux pump with larger magnitude of chemical collections within a standard normal laboratory setup. Two molecules were studied in detail for characterization. These molecules can be further studied for preclinical trials in animals.

4.3 Future direction

The study led to the discovery of two novel chemical entities for combating multidrug resistance. IITR06144 is a broad spectrum antibacterial molecule and IITR08027 is an efflux pump inhibitor (EPIs) against the multidrug transporter, AbeM in *A. baumannii*. Both of these molecules have enormous possibility of commercial use against bacterial infections. However, a detailed further investigation is required for sending these molecules form laboratory to clinic. Both of these molecules have to be gone though different level of preclinical trials in animals. If they satisfy all the criteria, they can be used commercially. Effect of IITR0827 on other families of efflux pumps can also be investigated. A detailed structural analysis of both the molecules can be done to identify the functional region of the molecules interact with the target. Based on the structure-activity relation, these molecules can be truncated to more effective small structures for commercial use.

REFERENCES

REFERENCES

[1] S. J. Projan and D. M. Shlaes, "Antibacterial drug discovery: is it all downhill from here?," *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.*, vol. 10 Suppl 4, pp. 18–22, Nov. 2004.

[2] K. M. Overbye and J. F. Barrett, "Antibiotics: Where did we go wrong?," *Drug Discov. Today*, vol. 10, no. 1, pp. 45–52, Jan. 2005.

[3] M. N. Gwynn, A. Portnoy, S. F. Rittenhouse, and D. J. Payne, "Challenges of antibacterial discovery revisited," *Ann. N. Y. Acad. Sci.*, vol. 1213, pp. 5–19, Dec. 2010.

[4] S. E. Damodaran and S. Madhan, "Telavancin: A novel lipoglycopeptide antibiotic," *J. Pharmacol. Pharmacother.*, vol. 2, no. 2, pp. 135–137, 2011.

[5] M. A. Fischbach and C. T. Walsh, "Antibiotics for Emerging Pathogens," *Science*, vol. 325, no. 5944, pp. 1089–1093, Aug. 2009.

[6] H. W. Boucher, G. H. Talbot, D. K. Benjamin, J. Bradley, R. J. Guidos, R. N. Jones, B. E. Murray, R. A. Bonomo, and D. Gilbert, "10 × '20 Progress—Development of New Drugs Active Against Gram-Negative Bacilli: An Update From the Infectious Diseases Society of America," *Clin. Infect. Dis.*, Apr. 2013.

[7] P.-E. Fournier, D. Vallenet, V. Barbe, S. Audic, H. Ogata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult, and J.-M. Claverie, "Comparative genomics of multidrug resistance in Acinetobacter baumannii," *PLoS Genet.*, vol. 2, no. 1, p. e7, Jan. 2006.

[8] N. K. Sharma, R. Garg, S. Baliga, and G. Bhat K, "Nosocomial Infections and Drug Susceptibility Patterns in Methicillin Sensitive and Methicillin Resistant Staphylococcus aureus," *J. Clin. Diagn. Res. JCDR*, vol. 7, no. 10, pp. 2178–2180, Oct. 2013.

[9] P. D. Lister, D. J. Wolter, and N. D. Hanson, "Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms," *Clin. Microbiol. Rev.*, vol. 22, no. 4, pp. 582–610, Oct. 2009.

[10] P. K. Linden, "Treatment options for vancomycin-resistant enterococcal infections," *Drugs*, vol. 62, no. 3, pp. 425–441, 2002.

[11] A. Elemam, J. Rahimian, and W. Mandell, “Infection with panresistant Klebsiella pneumoniae: a report of 2 cases and a brief review of the literature,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 49, no. 2, pp. 271–274, Jul. 2009.

[12] G. V. Sanchez, R. N. Master, R. B. Clark, M. Fyyaz, P. Duvvuri, G. Ekta, and J. Bordon, “*Klebsiella pneumoniae* Antimicrobial Drug Resistance, United States, 1998–2010,” *Emerg. Infect. Dis.*, vol. 19, no. 1, pp. 133–136, Jan. 2013.

[13] I. K. Neonakis, G. Samonis, H. Messaritakis, S. Baritaki, A. Georgiladakis, S. Maraki, and D. A. Spandidos, “Resistance status and evolution trends of *Klebsiella pneumoniae* isolates in a university hospital in Greece: ineffectiveness of carbapenems and increasing resistance to colistin,” *Chemotherapy*, vol. 56, no. 6, pp. 448–452, 2010.

[14] H. Nikaido, “Multidrug resistance in bacteria,” *Annu. Rev. Biochem.*, vol. 78, pp. 119–146, 2009.

[15] L. J. V. Piddock, “Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria,” *Clin. Microbiol. Rev.*, vol. 19, no. 2, pp. 382–402, Apr. 2006.

[16] M. Putman, H. W. van Veen, and W. N. Konings, “Molecular Properties of Bacterial Multidrug Transporters,” *Microbiol. Mol. Biol. Rev.*, vol. 64, no. 4, pp. 672–693, Dec. 2000.

[17] L. Ejim, M. A. Farha, S. B. Falconer, J. Wildenhain, B. K. Coombes, M. Tyers, E. D. Brown, and G. D. Wright, “Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy,” *Nat. Chem. Biol.*, vol. 7, no. 6, pp. 348–350, Jun. 2011.

[18] F. Van Bambeke, J.-M. Pagès, and V. J. Lee, “Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux,” *Recent Patents Anti-Infect. Drug Disc.*, vol. 1, no. 2, pp. 157–175, Jun. 2006.

[19] B. R. Stockwell, “Chemical genetics: ligand-based discovery of gene function,” *Nat. Rev. Genet.*, vol. 1, no. 2, pp. 116–125, Nov. 2000.

[20] R. S. Lokey, “Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia,” *Curr. Opin. Chem. Biol.*, vol. 7, no. 1, pp. 91–96, Feb. 2003.

[21] E. D. Brown and G. D. Wright, “New targets and screening approaches in antimicrobial drug discovery,” *Chem. Rev.*, vol. 105, no. 2, pp. 759–774, Feb. 2005.

[22] A. Higashitani, N. Higashitani, and K. Horiuchi, “A cell division inhibitor SulA of *Escherichia coli* directly interacts with FtsZ through GTP hydrolysis,” *Biochem. Biophys. Res. Commun.*, vol. 209, no. 1, pp. 198–204, Apr. 1995.

[23] H. M. Geysen, F. Schoenen, D. Wagner, and R. Wagner, “Combinatorial compound libraries for drug discovery: an ongoing challenge,” *Nat. Rev. Drug Discov.*, vol. 2, no. 3, pp. 222–230, Mar. 2003.

[24] W. R. J. D. Galloway, A. Bender, M. Welch, and D. R. Spring, “The discovery of antibacterial agents using diversity-oriented synthesis,” *Chem. Commun.*, no. 18, pp. 2446–2462, Apr. 2009.

[25] P. Gribbon and A. Sewing, “High-throughput drug discovery: what can we expect from HTS?,” *Drug Discov. Today*, vol. 10, no. 1, pp. 17–22, Jan. 2005.

[26] L. M. Mayr and D. Bojanic, “Novel trends in high-throughput screening,” *Curr. Opin. Pharmacol.*, vol. 9, no. 5, pp. 580–588, Oct. 2009.

[27] E. D. Brown, “Screening in academe: a perspective on implementation of university-based small molecule screening,” *J. Biomol. Screen.*, vol. 8, no. 4, pp. 377–379, Aug. 2003.

[28] K. P. Mishra, L. Ganju, M. Sairam, P. K. Banerjee, and R. C. Sawhney, “A review of high throughput technology for the screening of natural products,” *Biomed. Pharmacother. Biomédé. Pharmacothérapie*, vol. 62, no. 2, pp. 94–98, Feb. 2008.

[29] R. Pathania and E. D. Brown, “Small and lethal: searching for new antibacterial compounds with novel modes of action,” *Biochem. Cell Biol. Biochim. Biol. Cell.*, vol. 86, no. 2, pp. 111–115, Apr. 2008.

[30] O. Lomovskaya, M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee, “Identification and Characterization of Inhibitors of Multidrug Resistance Efflux Pumps in Pseudomonas aeruginosa: Novel Agents for Combination Therapy,” *Antimicrob. Agents Chemother.*, vol. 45, no. 1, pp. 105–116, Jan. 2001.

[31] J. Klekota, E. Brauner, and S. L. Schreiber, “Identifying biologically active compound classes using phenotypic screening data and sampling statistics,” *J. Chem. Inf. Model.*, vol. 45, no. 6, pp. 1824–1836, Dec. 2005.

[32] M. M. K. Jayasekera, K. Onheiber, J. Keith, H. Venkatesan, A. Santillan, E. M. Stocking, L. Tang, J. Miller, L. Gomez, B. Rhead, T. Delcamp, S. Huang, R. Wolin, E. V. Bobkova, and K. J. Shaw, "Identification of novel inhibitors of bacterial translation elongation factors," *Antimicrob. Agents Chemother.*, vol. 49, no. 1, pp. 131–136, Jan. 2005.

[33] E. André, L. Bastide, S. Michaux-Charachon, A. Gouby, P. Villain-Guillot, J. Latouche, A. Bouchet, M. Gualtiéri, and J.-P. Leonetti, "Novel synthetic molecules targeting the bacterial RNA polymerase assembly," *J. Antimicrob. Chemother.*, vol. 57, no. 2, pp. 245–251, Feb. 2006.

[34] D. J. Haydon, N. R. Stokes, R. Ure, G. Galbraith, J. M. Bennett, D. R. Brown, P. J. Baker, V. V. Barynin, D. W. Rice, S. E. Sedelnikova, J. R. Heal, J. M. Sheridan, S. T. Aiwale, P. K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, and L. G. Czaplewski, "An inhibitor of FtsZ with potent and selective anti-staphylococcal activity," *Science*, vol. 321, no. 5896, pp. 1673–1675, Sep. 2008.

[35] R. Pathania, S. Zlitni, C. Barker, R. Das, D. A. Gerritsma, J. Lebert, E. Awuah, G. Melacini, F. A. Capretta, and E. D. Brown, "Chemical genomics in Escherichia coli identifies an inhibitor of bacterial lipoprotein targeting," *Nat. Chem. Biol.*, vol. 5, no. 11, pp. 849–856, Nov. 2009.

[36] L. A. Lieberman and D. E. Higgins, "A small-molecule screen identifies the antipsychotic drug pimozide as an inhibitor of Listeria monocytogenes infection," *Antimicrob. Agents Chemother.*, vol. 53, no. 2, pp. 756–764, Feb. 2009.

[37] J. G. Swoboda, T. C. Meredith, J. Campbell, S. Brown, T. Suzuki, T. Bollenbach, A. J. Malhowski, R. Kishony, M. S. Gilmore, and S. Walker, "Discovery of a Small Molecule that Blocks Wall Teichoic Acid Biosynthesis in Staphylococcus aureus," *ACS Chem. Biol.*, vol. 4, no. 10, pp. 875–883, Oct. 2009.

[38] J. Huber, R. G. K. Donald, S. H. Lee, L. W. Jarantow, M. J. Salvatore, X. Meng, R. Painter, R. H. Onishi, J. Occi, K. Dorso, K. Young, Y. W. Park, S. Skwish, M. J. Szymonifka, T. S. Waddell, L. Miesel, J. W. Phillips, and T. Roemer, "Chemical genetic identification of peptidoglycan inhibitors potentiating carbapenem activity against methicillin-resistant Staphylococcus aureus," *Chem. Biol.*, vol. 16, no. 8, pp. 837–848, Aug. 2009.

[39] S. G. Richter, D. Elli, H. K. Kim, A. P. A. Hendrickx, J. A. Sorg, O. Schneewind, and D. Missiakas, “Small molecule inhibitor of lipoteichoic acid synthesis is an antibiotic for Gram-positive bacteria,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 9, pp. 3531–3536, Feb. 2013.

[40] S. J. Projan, “Why is big Pharma getting out of antibacterial drug discovery?,” *Curr. Opin. Microbiol.*, vol. 6, no. 5, pp. 427–430, Oct. 2003.

[41] A. E. Belanger, A. Lai, M. A. Brackman, and D. J. LeBlanc, “PCR-Based Ordered Genomic Libraries: a New Approach to Drug Target Identification for Streptococcus pneumoniae,” *Antimicrob. Agents Chemother.*, vol. 46, no. 8, pp. 2507–2512, Aug. 2002.

[42] B. Hutter, C. Schaab, S. Albrecht, M. Borgmann, N. A. Brunner, C. Freiberg, K. Ziegelbauer, C. O. Rock, I. Ivanov, and H. Loferer, “Prediction of Mechanisms of Action of Antibacterial Compounds by Gene Expression Profiling,” *Antimicrob. Agents Chemother.*, vol. 48, no. 8, pp. 2838–2844, Aug. 2004.

[43] X. Li, M. Zolli-Juran, J. D. Cechetto, D. M. Daigle, G. D. Wright, and E. D. Brown, “Multicopy suppressors for novel antibacterial compounds reveal targets and drug efflux susceptibility,” *Chem. Biol.*, vol. 11, no. 10, pp. 1423–1430, Oct. 2004.

[44] A. Liu, L. Tran, E. Becket, K. Lee, L. Chinn, E. Park, K. Tran, and J. H. Miller, “Antibiotic Sensitivity Profiles Determined with an Escherichia coli Gene Knockout Collection: Generating an Antibiotic Bar Code,” *Antimicrob. Agents Chemother.*, vol. 54, no. 4, pp. 1393–1403, Apr. 2010.

[45] J. M. Boberek, J. Stach, and L. Good, “Genetic Evidence for Inhibition of Bacterial Division Protein FtsZ by Berberine,” *PLoS ONE*, vol. 5, no. 10, p. e13745, Oct. 2010.

[46] S. D. Patil, R. Sharma, S. Srivastava, N. K. Navani, and R. Pathania, “Downregulation of yidC in *Escherichia coli* by antisense RNA expression results in sensitization to antibacterial essential oils eugenol and carvacrol,” *PloS One*, vol. 8, no. 3, p. e57370, 2013.

[47] H. Wang, D. Claveau, J. P. Vaillancourt, T. Roemer, and T. C. Meredith, “High-frequency transposition for determining antibacterial mode of action,” *Nat. Chem. Biol.*, vol. 7, no. 10, pp. 720–729, Oct. 2011.

[48] N. Hever and S. Belkin, “A Dual-Color Bacterial Reporter Strain for the Detection of Toxic and Genotoxic Effects,” *Eng. Life Sci.*, vol. 6, no. 3, pp. 319–323, 2006.

[49] T. Rosenberger, J. K. Brülle, and P. Sander, “A β-Lactamase Based Reporter System for ESX Dependent Protein Translocation in *Mycobacteria*,” *PLoS ONE*, vol. 7, no. 4, p. e35453, Apr. 2012.

[50] S. B. Falconer, T. L. Czarny, and E. D. Brown, “Antibiotics as probes of biological complexity,” *Nat. Chem. Biol.*, vol. 7, no. 7, pp. 415–423, Jul. 2011.

[51] M. A. Kohanski, D. J. Dwyer, B. Hayete, C. A. Lawrence, and J. J. Collins, “A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics,” *Cell*, vol. 130, no. 5, pp. 797–810, Jul. 2007.

[52] S. S. Justice, D. A. Hunstad, L. Cegelski, and S. J. Hultgren, “Morphological plasticity as a bacterial survival strategy,” *Nat. Rev. Microbiol.*, vol. 6, no. 2, pp. 162–168, Feb. 2008.

[53] E. Harry, L. Monahan, and L. Thompson, “Bacterial cell division: the mechanism and its precison,” *Int. Rev. Cytol.*, vol. 253, pp. 27–94, 2006.

[54] O. Huisman, R. D’Ari, and S. Gottesman, “Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 81, no. 14, pp. 4490–4494, Jul. 1984.

[55] T. M. Hill, B. Sharma, M. Valjavec-Gratian, and J. Smith, “sfi-independent filamentation in *Escherichia coli* Is lexA dependent and requires DNA damage for induction,” *J. Bacteriol.*, vol. 179, no. 6, pp. 1931–1939, Mar. 1997.

[56] L. J. Wu and J. Errington, “Nucleoid occlusion and bacterial cell division,” *Nat. Rev. Microbiol.*, vol. 10, no. 1, pp. 8–12, Jan. 2012.

[57] H. Cho, H. R. McManus, S. L. Dove, and T. G. Bernhardt, “Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 9, pp. 3773–3778, Mar. 2011.

[58] E. Bi and J. Lutkenhaus, “Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring,” *J. Bacteriol.*, vol. 175, no. 4, pp. 1118–1125, Feb. 1993.

[59] L. I. Rothfield and S. S. Justice, “Bacterial cell division: the cycle of the ring,” *Cell*, vol. 88, no. 5, pp. 581–584, Mar. 1997.

[60] C. A. Hale, A. C. Rhee, and P. A. de Boer, “ZipA-induced bundling of FtsZ polymers mediated by an interaction between C-terminal domains,” *J. Bacteriol.*, vol. 182, no. 18, pp. 5153–5166, Sep. 2000.

[61] G. L. Kedderis and G. T. Miwa, “The metabolic activation of nitroheterocyclic therapeutic agents,” *Drug Metab. Rev.*, vol. 19, no. 1, pp. 33–62, 1988.

[62] D. I. Edwards, “Mechanisms of selective toxicity of metronidazole and other nitroimidazole drugs.,” *Br. J. Vener. Dis.*, vol. 56, no. 5, pp. 285–90, 1980.

[63] Y. Tu and D. R. McCalla, “Effect of activated nitrofurans on DNA,” *Biochim. Biophys. Acta BBA - Nucleic Acids Protein Synth.*, vol. 402, no. 2, pp. 142–149, Aug. 1975.

[64] D. R. McCalla, P. Olive, Y. Tu, and M. L. Fan, “Nitrofurazone-reducing enzymes in E. coli and their role in drug activation in vivo,” *Can. J. Microbiol.*, vol. 21, no. 10, pp. 1484–1491, Oct. 1975.

[65] D. R. McCalla, P. Olive, Y. Tu, and M. L. Fan, “Nitrofurazone-reducing enzymes in E. coli and their role in drug activation in vivo,” *Can. J. Microbiol.*, vol. 21, no. 10, pp. 1484–1491, Oct. 1975.

[66] J. Whiteway, P. Koziarz, J. Veall, N. Sandhu, P. Kumar, B. Hoecher, and I. B. Lambert, “Oxygen-insensitive nitroreductases: analysis of the roles of nfsA and nfsB in development of resistance to 5-nitrofuran derivatives in Escherichia coli,” *J. Bacteriol.*, vol. 180, no. 21, pp. 5529–5539, Nov. 1998.

[67] L. Sandegren, A. Lindqvist, G. Kahlmeter, and D. I. Andersson, “Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*,” *J. Antimicrob. Chemother.*, vol. 62, no. 3, pp. 495–503, Sep. 2008.

[68] G. L. Kedderis and G. T. Miwa, “The metabolic activation of nitroheterocyclic therapeutic agents,” *Drug Metab. Rev.*, vol. 19, no. 1, pp. 33–62, 1988.

[69] B. P. Goldstein, R. R. Vidal-Plana, B. Cavalleri, L. Zerilli, G. Carniti, and L. G. Silvestri, “The mechanism of action of nitro-heterocyclic antimicrobial drugs. Metabolic activation by micro-organisms,” *J. Gen. Microbiol.*, vol. 100, no. 2, pp. 283–298, Jun. 1977.

[70] M. Kitagawa, T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori, "Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research," *DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes*, vol. 12, no. 5, pp. 291–299, 2005.

[71] S. Goh, J. M. Boberek, N. Nakashima, J. Stach, and L. Good, "Concurrent Growth Rate and Transcript Analyses Reveal Essential Gene Stringency in *Escherichia coli*," *PLoS ONE*, vol. 4, no. 6, p. e6061, Jun. 2009.

[72] M. A. Webber and L. J. V. Piddock, "The importance of efflux pumps in bacterial antibiotic resistance," *J. Antimicrob. Chemother.*, vol. 51, no. 1, pp. 9–11, Jan. 2003.

[73] A. K. Bhardwaj and P. Mohanty, "Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvinating the antimicrobial chemotherapy," *Recent Patents Anti-Infect. Drug Disc.*, vol. 7, no. 1, pp. 73–89, Apr. 2012.

[74] J. Sun, Z. Deng, and A. Yan, "Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations," *Biochem. Biophys. Res. Commun.*, May 2014.

[75] K. Poole, "Efflux-mediated antimicrobial resistance," *J. Antimicrob. Chemother.*, vol. 56, no. 1, pp. 20–51, Jul. 2005.

[76] K. Poole, "Efflux-mediated multiresistance in Gram-negative bacteria," *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.*, vol. 10, no. 1, pp. 12–26, Jan. 2004.

[77] D. C. Rees, E. Johnson, and O. Lewinson, "ABC transporters: the power to change," *Nat. Rev. Mol. Cell Biol.*, vol. 10, no. 3, pp. 218–227, Mar. 2009.

[78] J. C. Malinverni and T. J. Silhavy, "An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 19, pp. 8009–8014, May 2009.

[79] P. Wieczorek, P. Sacha, T. Hauschild, M. Zórawski, M. Krawczyk, and E. Tryniszewska, "Multidrug resistant *Acinetobacter baumannii*--the role of AdeABC (RND family) efflux pump in resistance to antibiotics," *Folia Histochem. Cytobiol. Pol. Acad. Sci. Pol. Histochem. Cytochem. Soc.*, vol. 46, no. 3, pp. 257–267, 2008.

[80] L. Damier-Piolle, S. Magnet, S. Brémont, T. Lambert, and P. Courvalin, “AdeIJK, a Resistance-Nodulation-Cell Division Pump Effluxing Multiple Antibiotics in *Acinetobacter baumannii*,” *Antimicrob. Agents Chemother.*, vol. 52, no. 2, pp. 557–562, Feb. 2008.

[81] S. S. Pao, I. T. Paulsen, and M. H. Saier, “Major facilitator superfamily,” *Microbiol. Mol. Biol. Rev. MMBR*, vol. 62, no. 1, pp. 1–34, Mar. 1998.

[82] I. T. Paulsen, M. H. Brown, and R. A. Skurray, “Proton-dependent multidrug efflux systems.,” *Microbiol. Rev.*, vol. 60, no. 4, pp. 575–608, Dec. 1996.

[83] V. B. Srinivasan, G. Rajamohan, and W. A. Gebreyes, “Role of AbeS, a Novel Efflux Pump of the SMR Family of Transporters, in Resistance to Antimicrobial Agents in *Acinetobacter baumannii*,” *Antimicrob. Agents Chemother.*, vol. 53, no. 12, pp. 5312–5316, Dec. 2009.

[84] I. T. Paulsen, R. A. Skurray, R. Tam, M. H. Saier, R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius, “The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs,” *Mol. Microbiol.*, vol. 19, no. 6, pp. 1167–1175, Mar. 1996.

[85] T. Kuroda and T. Tsuchiya, “Multidrug efflux transporters in the MATE family,” *Biochim. Biophys. Acta BBA - Proteins Proteomics*, vol. 1794, no. 5, pp. 763–768, May 2009.

[86] X.-Z. Su, J. Chen, T. Mizushima, T. Kuroda, and T. Tsuchiya, “AbeM, an H+-Coupled *Acinetobacter baumannii* Multidrug Efflux Pump Belonging to the MATE Family of Transporters,” *Antimicrob. Agents Chemother.*, vol. 49, no. 10, pp. 4362–4364, Oct. 2005.

[87] F. Long, C. Rouquette-Loughlin, W. M. Shafer, and E. W. Yu, “Functional cloning and characterization of the multidrug efflux pumps NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli*,” *Antimicrob. Agents Chemother.*, vol. 52, no. 9, pp. 3052–3060, Sep. 2008.

[88] J. Chen, Y. Morita, M. N. Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya, “VmrA, a member of a novel class of Na(+)-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*,” *J. Bacteriol.*, vol. 184, no. 2, pp. 572–576, Jan. 2002.

[89] Y. Morita, K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima, and T. Tsuchiya, "NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*," *Antimicrob. Agents Chemother.*, vol. 42, no. 7, pp. 1778–1782, Jul. 1998.

[90] W. Ogawa, Y. Minato, H. Dodan, M. Onishi, T. Tsuchiya, and T. Kuroda, "Characterization of MATE-type multidrug efflux pumps from *Klebsiella pneumoniae* MGH78578," *PloS One*, vol. 10, no. 3, p. e0121619, 2015.

[91] F. McAleese, P. Petersen, A. Ruzin, P. M. Dunman, E. Murphy, S. J. Projan, and P. A. Bradford, "A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived Staphylococcus aureus mutants to tigecycline," *Antimicrob. Agents Chemother.*, vol. 49, no. 5, pp. 1865–1871, May 2005.

[92] A. Y. Peleg, H. Seifert, and D. L. Paterson, "*Acinetobacter baumannii*: emergence of a successful pathogen," *Clin. Microbiol. Rev.*, vol. 21, no. 3, pp. 538–582, Jul. 2008.

[93] A. Howard, M. O'Donoghue, A. Feeney, and R. D. Sleator, "*Acinetobacter baumannii*: an emerging opportunistic pathogen," *Virulence*, vol. 3, no. 3, pp. 243–250, May 2012.

[94] S. Coyne, P. Courvalin, and B. Périchon, "Efflux-mediated antibiotic resistance in *Acinetobacter spp*," *Antimicrob. Agents Chemother.*, vol. 55, no. 3, pp. 947–953, Mar. 2011.

[95] M. Van Looveren, H. Goossens, and ARPAC Steering Group, "Antimicrobial resistance of *Acinetobacter spp.* in Europe," *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.*, vol. 10, no. 8, pp. 684–704, Aug. 2004.

[96] A. C. Opazo, S. M. Mella, M. Y. Domínguez, H. T. Bello, and G. R. González, "[Multi-drug efflux pumps and antibiotic resistance in *Acinetobacter baumannii*]," *Rev. Chil. Infectol. Órgano Of. Soc. Chil. Infectol.*, vol. 26, no. 6, pp. 499–503, Dec. 2009.

[97] L. Lin, B.-D. Ling, and X.-Z. Li, "Distribution of the multidrug efflux pump genes, *adeABC, adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex," *Int. J. Antimicrob. Agents*, vol. 33, no. 1, pp. 27–32, Jan. 2009.

[98] G. Rajamohan, V. B. Srinivasan, and W. A. Gebreyes, "Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant

resistance in *Acinetobacter baumannii*," *J. Antimicrob. Chemother.*, vol. 65, no. 9, pp. 1919–1925, Sep. 2010.

[99] B. Marquez, "Bacterial efflux systems and efflux pumps inhibitors," *Biochimie*, vol. 87, no. 12, pp. 1137–1147, Dec. 2005.

[100] F. M. MacKenzie and I. M. Gould, "The post-antibiotic effect," *J. Antimicrob. Chemother.*, vol. 32, no. 4, pp. 519–537, Oct. 1993.

[101] A. Athamna, M. Athamna, B. Medlej, D. J. Bast, and E. Rubinstein, "In vitro post-antibiotic effect of fluoroquinolones, macrolides, beta-lactams, tetracyclines, vancomycin, clindamycin, linezolid, chloramphenicol, quinupristin/dalfopristin and rifampicin on Bacillus anthracis," *J. Antimicrob. Chemother.*, vol. 53, no. 4, pp. 609–615, Apr. 2004.

[102] S. Baugh, C. R. Phillips, A. S. Ekanayaka, L. J. V. Piddock, and M. A. Webber, "Inhibition of multidrug efflux as a strategy to prevent biofilm formation," *J. Antimicrob. Chemother.*, vol. 69, no. 3, pp. 673–681, Mar. 2014.

[103] T. J. Opperman, S. M. Kwasny, H.-S. Kim, S. T. Nguyen, C. Houseweart, S. D'Souza, G. C. Walker, N. P. Peet, H. Nikaido, and T. L. Bowlin, "Characterization of a novel pyranopyridine inhibitor of the AcrAB efflux pump of Escherichia coli," *Antimicrob. Agents Chemother.*, vol. 58, no. 2, pp. 722–733, 2014.

[104] A. Kumar, I. A. Khan, S. Koul, J. L. Koul, S. C. Taneja, I. Ali, F. Ali, S. Sharma, Z. M. Mirza, M. Kumar, P. L. Sangwan, P. Gupta, N. Thota, and G. N. Qazi, "Novel structural analogues of piperine as inhibitors of the NorA efflux pump of *Staphylococcus aureus*," *J. Antimicrob. Chemother.*, vol. 61, no. 6, pp. 1270–1276, Jun. 2008.

[105] T. E. Renau, R. Léger, E. M. Flamme, J. Sangalang, M. W. She, R. Yen, C. L. Gannon, D. Griffith, S. Chamberland, O. Lomovskaya, S. J. Hecker, V. J. Lee, T. Ohta, and K. Nakayama, "Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin," *J. Med. Chem.*, vol. 42, no. 24, pp. 4928–4931, Dec. 1999.

[106] O. Lomovskaya, M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee, "Identification and characterization of inhibitors of multidrug resistance efflux pumps in

Pseudomonas aeruginosa: novel agents for combination therapy," *Antimicrob. Agents Chemother.*, vol. 45, no. 1, pp. 105–116, Jan. 2001.

[107] M. L. Marchetti, J. Errecalde, and N. Mestorino, "Effect of 1-(1-naphthylmethyl)-piperazine on antimicrobial agent susceptibility in multidrug-resistant isogenic and veterinary *Escherichia coli* field strains," *J. Med. Microbiol.*, vol. 61, no. Pt 6, pp. 786–792, Jun. 2012.

[108] J. A. Bohnert and W. V. Kern, "Selected arylpiperazines are capable of reversing multidrug resistance in Escherichia coli overexpressing RND efflux pumps," *Antimicrob. Agents Chemother.*, vol. 49, no. 2, pp. 849–852, Feb. 2005.

[109] S. Gibbons, M. Oluwatuyi, and G. W. Kaatz, "A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*," *J. Antimicrob. Chemother.*, vol. 51, no. 1, pp. 13–17, Jan. 2003.

[110] S. Gupta, K. A. Cohen, K. Winglee, M. Maiga, B. Diarra, and W. R. Bishai, "Efflux inhibition with verapamil potentiates bedaquiline in *Mycobacterium tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 58, no. 1, pp. 574–576, 2014.

[111] C. Blanchard, P. Barnett, J. Perlmutter, and P. M. Dunman, "Identification of *Acinetobacter baumannii* serum-associated antibiotic efflux pump inhibitors," *Antimicrob. Agents Chemother.*, vol. 58, no. 11, pp. 6360–6370, Nov. 2014.

[112] M. Stavri, L. J. V. Piddock, and S. Gibbons, "Bacterial efflux pump inhibitors from natural sources," *J. Antimicrob. Chemother.*, vol. 59, no. 6, pp. 1247–1260, Jun. 2007.

[113] D. Lechner, S. Gibbons, and F. Bucar, "Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*," *J. Antimicrob. Chemother.*, vol. 62, no. 2, pp. 345–348, Aug. 2008.

[114] J. Jin, J.-Y. Zhang, N. Guo, H. Sheng, L. Li, J.-C. Liang, X.-L. Wang, Y. Li, M.-Y. Liu, X.-P. Wu, and L. Yu, "Farnesol, a potential efflux pump inhibitor in *Mycobacterium smegmatis*," *Mol. Basel Switz.*, vol. 15, no. 11, pp. 7750–7762, Nov. 2010.

[115] S. K. Roy, N. Kumari, S. Pahwa, U. C. Agrahari, K. K. Bhutani, S. M. Jachak, and H. Nandanwar, "NorA efflux pump inhibitory activity of coumarins from Mesua ferrea," *Fitoterapia*, vol. 90, pp. 140–150, Oct. 2013.

[116] S. K. Roy, S. Pahwa, H. Nandanwar, and S. M. Jachak, “Phenylpropanoids of Alpinia galanga as efflux pump inhibitors in *Mycobacterium smegmatis.* mc^2 155,” *Fitoterapia*, vol. 83, no. 7, pp. 1248–1255, Oct. 2012.

[117] S. K. Roy, N. Kumari, S. Gupta, S. Pahwa, H. Nandanwar, and S. M. Jachak, “7-Hydroxy-(E)-3-phenylmethylene-chroman-4-one analogues as efflux pump inhibitors against *Mycobacterium smegmatis* mc^2 155,” *Eur. J. Med. Chem.*, vol. 66, pp. 499–507, Aug. 2013.

[118] Z. M. Mirza, A. Kumar, N. P. Kalia, A. Zargar, and I. A. Khan, “Piperine as an inhibitor of the MdeA efflux pump of *Staphylococcus aureus*,” *J. Med. Microbiol.*, vol. 60, no. Pt 10, pp. 1472–1478, Oct. 2011.

[119] S. Sharma, M. Kumar, S. Sharma, A. Nargotra, S. Koul, and I. A. Khan, “Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of *Mycobacterium tuberculosis*,” *J. Antimicrob. Chemother.*, vol. 65, no. 8, pp. 1694–1701, Aug. 2010.

[120] N. P. Kalia, P. Mahajan, R. Mehra, A. Nargotra, J. P. Sharma, S. Koul, and I. A. Khan, “Capsaicin, a novel inhibitor of the NorA efflux pump, reduces the intracellular invasion of *Staphylococcus aureus*,” *J. Antimicrob. Chemother.*, vol. 67, no. 10, pp. 2401–2408, Oct. 2012.

[121] K. M. Hujer, A. M. Hujer, A. Endimiani, J. M. Thomson, M. D. Adams, K. Goglin, P. N. Rather, T.-T. D. Pennella, C. Massire, M. W. Eshoo, R. Sampath, L. B. Blyn, D. J. Ecker, and R. A. Bonomo, “Rapid determination of quinolone resistance in Acinetobacter spp,” *J. Clin. Microbiol.*, vol. 47, no. 5, pp. 1436–1442, May 2009.

[122] S. Park, K. M. Lee, Y. S. Yoo, J. S. Yoo, J. I. Yoo, H. S. Kim, Y. S. Lee, and G. T. Chung, “Alterations of *gyrA, gyrB,* and *parC* and Activity of Efflux Pump in Fluoroquinolone-resistant *Acinetobacter baumannii*,” *Osong Public Health Res. Perspect.*, vol. 2, no. 3, pp. 164–170, Dec. 2011.

[123] S. Chopra and A. Galande, “A fluoroquinolone-resistant *Acinetobacter baumannii* without the quinolone resistance-determining region mutations,” *J. Antimicrob. Chemother.*, p. dkr364, Sep. 2011.

[124] K. Poole, “Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria,” *Antimicrob. Agents Chemother.*, vol. 44, no. 9, pp. 2233–2241, Sep. 2000.

[125] S. Magnet, P. Courvalin, and T. Lambert, “Resistance-Nodulation-Cell Division-Type Efflux Pump Involved in Aminoglycoside Resistance in *Acinetobacter baumannii* Strain BM4454,” *Antimicrob. Agents Chemother.*, vol. 45, no. 12, pp. 3375–3380, Dec. 2001.

[126] L. Zhang, X. Z. Li, and K. Poole, “Fluoroquinolone susceptibilities of efflux-mediated multidrug-resistant *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Burkholderia cepacia*,” *J. Antimicrob. Chemother.*, vol. 48, no. 4, pp. 549–552, Oct. 2001.

[127] A. J. Baylay and L. J. V. Piddock, “Clinically relevant fluoroquinolone resistance due to constitutive overexpression of the PatAB ABC transporter in *Streptococcus pneumoniae* is conferred by disruption of a transcriptional attenuator,” *J. Antimicrob. Chemother.*, vol. 70, no. 3, pp. 670–679, Mar. 2015.

[128] F. El Garch, A. Lismond, L. J. V. Piddock, P. Courvalin, P. M. Tulkens, and F. Van Bambeke, “Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*,” *J. Antimicrob. Chemother.*, vol. 65, no. 10, pp. 2076–2082, Oct. 2010.

[129] S. K. Banerjee, K. Bhatt, S. Rana, P. Misra, and P. K. Chakraborti, “Involvement of an efflux system in mediating high level of fluoroquinolone resistance in *Mycobacterium smegmatis*,” *Biochem. Biophys. Res. Commun.*, vol. 226, no. 2, pp. 362–368, Sep. 1996.

[130] S. K. Banerjee, P. Misra, K. Bhatt, S. C. Mande, and P. K. Chakraborti, “Identification of an ABC transporter gene that exhibits mRNA level overexpression in fluoroquinolone-resistant *Mycobacterium smegmatis*,” *FEBS Lett.*, vol. 425, no. 1, pp. 151–156, Mar. 1998.

[131] P. K. Chakraborti, K. Bhatt, S. K. Banerjee, and P. Misra, “Role of an ABC importer in mycobacterial drug resistance,” *Biosci. Rep.*, vol. 19, no. 4, pp. 293–300, Aug. 1999.

[132] G. Orhan, A. Bayram, Y. Zer, and I. Balci, “Synergy tests by E test and checkerboard methods of antimicrobial combinations against Brucella melitensis,” *J. Clin. Microbiol.*, vol. 43, no. 1, pp. 140–143, Jan. 2005.

[133] T. Mine, Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya, “Evidence for chloramphenicol/H+ antiport in Cmr (MdfA) system of *Escherichia coli* and properties of the antiporter,” *J. Biochem. (Tokyo)*, vol. 124, no. 1, pp. 187–193, Jul. 1998.

[134] B. P. Rosen and J. S. McClees, “Active transport of calcium in inverted membrane vesicles of *Escherichia coli*,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 71, no. 12, pp. 5042–5046, Dec. 1974.

[135] E. S. Lennox, “Transduction of linked genetic characters of the host by bacteriophage P1,” *Virology*, vol. 1, no. 2, pp. 190–206, Jul. 1955.

[136] T. Kuroda, T. Shimamoto, K. Inaba, M. Tsuda, and T. Tsuchiya, “Properties and sequence of the NhaA Na+/H+ antiporter of *Vibrio parahaemolyticus*,” *J. Biochem. (Tokyo)*, vol. 116, no. 5, pp. 1030–1038, Nov. 1994.

[137] G.-X. He, C. Thorpe, D. Walsh, R. Crow, H. Chen, S. Kumar, and M. F. Varela, “EmmdR, a new member of the MATE family of multidrug transporters, extrudes quinolones from *Enterobacter cloacae*,” *Arch. Microbiol.*, vol. 193, no. 10, pp. 759–765, Oct. 2011.

[138] G. A. Pankuch, M. R. Jacobs, and P. C. Appelbaum, “Postantibiotic effects of daptomycin against 14 staphylococcal and pneumococcal clinical isolates,” *Antimicrob. Agents Chemother.*, vol. 47, no. 9, pp. 3012–3014, Sep. 2003.

[139] W. A. Craig and S. C. Ebert, “Killing and regrowth of bacteria in vitro: a review,” *Scand. J. Infect. Dis. Suppl.*, vol. 74, pp. 63–70, 1990.

[140] R. P. Smith, A. L. Baltch, P. B. Michelsen, W. J. Ritz, and R. Alteri, “Use of the microbial growth curve in postantibiotic effect studies of *Legionella pneumophila*,” *Antimicrob. Agents Chemother.*, vol. 47, no. 3, pp. 1081–1087, Mar. 2003.

[141] H. B. Drugeon, M. E. Juvin, and A. Bryskier, “Relative potential for selection of fluoroquinolone-resistant Streptococcus pneumoniae strains by levofloxacin: comparison with ciprofloxacin, sparfloxacin and ofloxacin,” *J. Antimicrob. Chemother.*, vol. 43 Suppl C, pp. 55–59, Jun. 1999.

[142] K. Nikhil, S. Sharan, A. Chakraborty, N. Bodipati, R. Krishna Peddinti, and P. Roy, “Role of isothiocyanate conjugate of pterostilbene on the inhibition of MCF-7 cell proliferation and tumor growth in Ehrlich ascitic cell induced tumor bearing mice,” *Exp. Cell Res.*, vol. 320, no. 2, pp. 311–328, Jan. 2014.

[143] H. Yoneyama and R. Katsumata, “Antibiotic resistance in bacteria and its future for novel antibiotic development,” *Biosci. Biotechnol. Biochem.*, vol. 70, no. 5, pp. 1060–1075, May 2006.

[144] N. Rajpara, A. Patel, N. Tiwari, J. Bahuguna, A. Antony, I. Choudhury, A. Ghosh, R. Jain, A. Ghosh, and A. K. Bhardwaj, “Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons,” *Int. J. Antimicrob. Agents*, vol. 34, no. 3, pp. 220–225, Sep. 2009.

[145] R. L. Lock and E. J. Harry, “Cell-division inhibitors: new insights for future antibiotics,” *Nat. Rev. Drug Discov.*, vol. 7, no. 4, pp. 324–338, Apr. 2008.

[146] D. J. Haydon, N. R. Stokes, R. Ure, G. Galbraith, J. M. Bennett, D. R. Brown, P. J. Baker, V. V. Barynin, D. W. Rice, S. E. Sedelnikova, J. R. Heal, J. M. Sheridan, S. T. Aiwale, P. K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, and L. G. Czaplewski, “An inhibitor of FtsZ with potent and selective anti-staphylococcal activity,” *Science*, vol. 321, no. 5896, pp. 1673–1675, Sep. 2008.

[147] N. R. Stokes, J. Sievers, S. Barker, J. M. Bennett, D. R. Brown, I. Collins, V. M. Errington, D. Foulger, M. Hall, R. Halsey, H. Johnson, V. Rose, H. B. Thomaides, D. J. Haydon, L. G. Czaplewski, and J. Errington, “Novel inhibitors of bacterial cytokinesis identified by a cell-based antibiotic screening assay,” *J. Biol. Chem.*, vol. 280, no. 48, pp. 39709–39715, Dec. 2005.

[148] P. N. Domadia, A. Bhunia, J. Sivaraman, S. Swarup, and D. Dasgupta, “Berberine targets assembly of *Escherichia coli* cell division protein FtsZ,” *Biochemistry (Mosc.)*, vol. 47, no. 10, pp. 3225–3234, Mar. 2008.

[149] P. Domadia, S. Swarup, A. Bhunia, J. Sivaraman, and D. Dasgupta, “Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde,” *Biochem. Pharmacol.*, vol. 74, no. 6, pp. 831–840, Sep. 2007.

[150] C. Schaffner-Barbero, M. Martín-Fontecha, P. Chacón, and J. M. Andreu, “Targeting the assembly of bacterial cell division protein FtsZ with small molecules,” *ACS Chem. Biol.*, vol. 7, no. 2, pp. 269–277, Feb. 2012.

[151] D. N. Margalit, L. Romberg, R. B. Mets, A. M. Hebert, T. J. Mitchison, M. W. Kirschner, and D. RayChaudhuri, “Targeting cell division: small-molecule inhibitors of FtsZ GTPase perturb cytokinetic ring assembly and induce bacterial lethality,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 32, pp. 11821–11826, Aug. 2004.

[152] M. A. Oliva, S. C. Cordell, and J. Löwe, “Structural insights into FtsZ protofilament formation,” *Nat. Struct. Mol. Biol.*, vol. 11, no. 12, pp. 1243–1250, Dec. 2004.

[153] J. Wang, A. Galgoci, S. Kodali, K. B. Herath, H. Jayasuriya, K. Dorso, F. Vicente, A. González, D. Cully, D. Bramhill, and S. Singh, “Discovery of a small molecule that inhibits cell division by blocking FtsZ, a novel therapeutic target of antibiotics,” *J. Biol. Chem.*, vol. 278, no. 45, pp. 44424–44428, Nov. 2003.

[154] S. Pichoff and J. Lutkenhaus, “Escherichia coli division inhibitor MinCD blocks septation by preventing Z-ring formation,” *J. Bacteriol.*, vol. 183, no. 22, pp. 6630–6635, Nov. 2001.

[155] B. P. Goldstein, R. R. Vidal-Plana, B. Cavalleri, L. Zerilli, G. Carniti, and L. G. Silvestri, “The mechanism of action of nitro-heterocyclic antimicrobial drugs. Metabolic activation by micro-organisms,” *J. Gen. Microbiol.*, vol. 100, no. 2, pp. 283–298, Jun. 1977.