Pharmacological and genetic studies on delayed death inhibitors of apicomplexan parasites

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DECLARATION

I, **Meenakshi Anil Belekar** , hereby declare that the work incorporated in the thesis and entitled "**Pharmacological and genetic studies on delayed death inhibitors of apicomplexan parasites**" submitted for the award of the Degree of **Doctor of Philosophy in Biological Sciences** to the **Academy of Scientific & Innovative Research (AcSIR)**, New Delhi, has been carried out by me at Division of Biological Sciences, CSIR-National Chemical Laboratory, Pune-411008, India, under the supervision of **Dr. Dhanasekaran Shanmugam**. The work is original and has not been submitted as a part or full by me for any degree or diploma to this or any other university. I further declare that the material obtained from other resources has been duly acknowledged in this thesis.

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Dedicated to my family and friends for their love and faith in me.....

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Meenakshi Anil Belekar

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List of Abbreviations

µM	Micro molar
AT	Adenine Thymine
ATCC	American Type Culture Collection
Atq	Atovaquone
BLE	Bleomycin
BP	Base pair
Cas	CRISPR associated systems
CAT	chloramphenicol acetyl transferase
cDNA	Complementary deoxyribonucleic acid
CLQ	Chloroquine
CMCM	Complete malaria culture media
CO2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4',6-diamidino-2-phenyllindole
DHFR-TS	Dihydrofolate reductase thymidylate synthase
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle's medium
DMEM	Dulbacco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribineuclic acid
dNTP	Deoxy ribonucleic acid
EC50	Effetive concentartion to kill half of the polpulation
EDTA	Ethylendiamine tetraacetate
ELISA	Enzyme-linked immune-sorbent assays
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein

GPCRs	G protein-coupled receptors
GSK	GlaxoSmithKline
HA	Hemagglutinin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HFF	Human foreskin fibroblast
hpi	Hours post infection
hr	Hour(s)
HRP2	Histidine rich protein 2
HSP70	Heat shock protein 70
HXGPRT	Hypoxanthin-xanthine-guanine phosphoribosyl transferase
iRBCs	Infected RBCs
KO	Knockout
LDH	Lactate dehydrogenase
LOPAC	Library of Pharmacologically Active Compounds
Luc	Luciferase
mM	Milli molar
MMV	Medicines for Malaria Venture, Geneva
MOA	Mechanism of action
nM	Nano molar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PFA	Paraformaldehyde
PV	Parasitophorous vacuole
PV	Parasitophorpous vacuole
PVM	Parasitophorpous vacuole membrane
Pyr	Pyrimethamine
qRT-PCR	Quantitative reverse-transcription PCR
RBCs	Red blood cells
RNA	Ribonucleic acid

rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute Media
SAG	Surface antigen
TATi	Tetracycline transactivator
TetR	Tetracycline repressor
Tg	*Toxoplasm gondii*
TPI-II	Triose phosphate isomerase II
UPRT	Uracil phosphoribosyl transferase
USA	United States of America
UTR	Untranslated region
wt	Wild type
YFP	Yellow fluorescent protein

Abstract

Discovering new drugs against apicomplexan parasites, preferably acting via novel mechanisms or extending our chemical toolkit for well determined targets are much needed. We have done detailed screening effort to identify novel inhibitors of *Toxoplasma gondii* using three different chemical libraries- "Sigma LOPAC", MMV "Malaria Box" and MMV "Pathogen Box".

Sigma LOPAC is a collection of 1280 bioactive small molecules from Sigma Aldrich and have known mechanism of action in mammalian system and these molecules could be used to identify some unusual and new druggable targets in apicomplexan parasites. The molecule collection from MMV 'Malaria Box' and 'Pathogen Box' includes ~800 chemically diverse small molecules in each set, with proven potency against malaria parasites as well as other infectious agents, however, through unknown mechanisms.

We first compared the anti-plasmodial and anti-toxoplasma activities of these libraries and found compounds with similar as well as mutually exclusive growth inhibitory effects and determined EC50 values for selected hits showing ≥80% growth inhibition at 10 μM. By comparing the EC50 values of *T. gondii* and *P. falciparum*, we identified a set of molecules that are effective with sub-micro molar potency against both parasites. This has allowed us to select molecules for detailed phenotypic studies in *T. gondii*, since it is similar in many aspects of cellular biology to malaria parasites, and possess corresponding orthologous genes. Using complementary phenotypic screens on *P. falciparum* and *T. gondii*, we differentiated sub-sets of molecules as inhibitors of overall cell growth of parasite, plastid segregation, parasite invasion, and egress, thereby providing early insights on their probable modes of action. These results highlight the advantage of comparative phenotypic screens in related species, as a means to identify lead molecules with conserved mode of action. Interestingly, few molecules that

failed to induce acute growth inhibition on *T. gondii* tachyzoites in a two-day proliferation assay caused delayed parasitic death upon extended exposure. At least 28 (4-Malaria Box, 23-Pathogen Box and 1-LOPAC) of these molecules appear to act by inducing apicoplast mis-segregation during daughter cell formation. We have prioritized these inhibitors for target identification and mechanistic studies. Further work on target identification and mechanism will facilitate developing anti-parasitic compounds which have novel chemical scaffolds and act via a very well-studied phenomenon of delayed death in these parasites. Collectively, our findings highlight the potential for chemical phenotype based identification of novel mechanisms and targets in related parasites, for the MMV molecules. It will help to develop a deeper understanding of novel targets that can be leveraged to develop the future generation of drugs with a wide spectrum of activity not just for malaria, but other related diseases as well.

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Publications

1. Gowtham Subramanian#, **Meenakshi A Belekar#**, Anurag Shukla, Jie Xin Tong, Ameya Sinha, Trang TT Chu, Akshay S. Kulkarni, Peter R Preiser, D. Srinivasa Reddy, Kevin SW Tan, Dhanasekaran Shanmugam and Rajesh Chandramohanadas; Targeted phenotypic screening in Plasmodium falciparum and Toxoplasma gondii reveals novel modes of action for MMV Malaria Box molecules; (*mSphere*. Jan 2018, 17;3(1).(# equal first author)

2. **Meenakshi A Belekar**, Tejashree Hingamire, Dhanasekaran Shanmugam. Phenotypic screen distinguish between the differential growth effect of bioactive molecules from Sigma-LOPAC on *Toxoplasma gondii* and *Plasmodium falciparum.* (To be communicated)

3. **Meenakshi A Belekar**, Tejashree Hingamire, Alok Tanala Patra, Olga Douvropoulou, Amit Kumar Subudhi, Raeece Naeem,Arnab Pain,Rajesh Chandramohandas, Dhanasekaran Shanmugam. Forward chemical-genetic screening on *T.gondii* gives an insight into molecular mechanism of delayed death phenotype using Pathogen Box molecules. (To be communicated)

4. **Patent:** Dumbala Srinivasa Reddy, Dhanasekaran Shanmugam, Remya Ramesh, Anurag Shukla, and **Meenakshi Anil Belekar**. A Pharmaceutical composition for preventing and treating malaria and toxoplasmosis. PCT/IN2017/050067 (WO) & 201611005767 (IN).

Chapter 1

Introduction of the apicomplexan parasites. Chemical and genetic screening in apicomplexan parasites and its impact on development of anti-parasitic drugs.

Introduction

Phylum apicomplexa comprises of thousands of unicellular eukaryotes, which are predominantly obligate intracellular parasites. The term apicomplexa was coined to indicate the presence of a complex of organelles at the apical end of these parasites, which facilitate parasite invasion and exit from infected host cells. *T. gondii* is one of the most promiscuous and successful parasite belonging to this phylum, and causes toxoplasmosis in humans and other warm blooded animals. In humans congenital toxoplasmosis can result in the development of toxoplasmosis-associated encephalitis (Luft *et al*., 1992). The *Plasmodium* genus is an important group comprised of parasites which cause malaria in humans and other animals. Malaria is responsible for at least half a million human deaths annually (World Malaria Report 2017). Other apicomplexan parasites which primarily infect animals include *Cryptosporidium* and *Cyclospora sp.,* which cause gastrointestinal enteritis and severe diarrhea (Tzipori *et al*., 2002); *Babesia* and *Theileria sp.,* which are transmitted *via* tick vectors and cause serious disease in a range of wild as well as domestic animals, including cattles and ultimately resulting in loss of productivity of these animals (Bishop *et al*., 2004); and *Eimeria sp.,* which causes coccidiosis in poultry resulting in significant economic loss worldwide (McDonald *et al*., 2009).

A wide range of hosts, ability to proliferate rapidly and potential for massive tissue destruction are the hallmark of pathogenesis induced by these pathogens in humans and animals. *Plasmodium sp*., are the most studied among these parasites, since malaria is a deadly human infectious disease affecting millions of people. The ability of these parasites to adapt well and survive unfavorable conditions is demonstrated by the rapidity with which these parasites become resistant to widely used antimalarial drugs. In case of *T. gondii*, the parasites can persist in the infected human hosts for their life-time as tissue cysts, especially in immunologically privileged tissues such as the brain where they can cause infection related

pathogenesis. Here again, the available drugs for treating toxoplasmosis are very few (example, clindamycin and sulfa drugs) and especially against the tissue cysts these are not very effective. Thus there is a need for identifying novel drug targets to develop new and better treatments against these parasitic diseases.

For this thesis research work, we have used *T. gondii* as the pathogen of choice for a number of reasons. First, this is an important human pathogen, which is present all over the world (unlike *Plasmodium*, which is restricted to tropical regions of the world due to constrain imposed by vector availability) and affects nearly 30% of the human population worldwide. Although infection by *T. gondii* is not fatal for a healthy adult human, it can be life threatening in immunologically weak hosts. Therefore, efforts on anti-toxoplasmosis drug discovery are still very relevant. Moreover, this parasite is also easily and safely maintained and propagated under laboratory conditions and a wide variety of molecular tools for genetic manipulation of this parasite is already available (Kim and Weiss *et al*., 2004). Moreover, *T. gondii* is also amenable for *in vivo* studies using mouse as the host. Finally, and most importantly, due to the shared evolutionary history of *T. gondii* with other apicomplexan parasites, a large number of genes are conserved as orthologs in these parasites. This is also reflected in the fact that many of the house keeping cellular functions, such as metabolism, are well conserved, and so inhibitors affecting these conserved processes can have pan-apicomplexan growth inhibiting effect. Thus drug mechanism of action (MOA) studies in one parasite (example, *T. gondii*) can help understand its effect on another parasite, such as *P. falciparum*. In fact *T. gondii* has been touted as a good model parasite to study cellular biology of apicomplexan parasites (Kim and Weiss *et al*., 2004).

The life cycle of T. gondii

The life cycle of *Toxoplasma* alternates between primary and secondary hosts (Dubey, 2008). The asexual development of *T. gondii* can occur in any warm-blooded animal, including humans, while only feline specie are known to support the sexual cycle (Dubey and Frenkel, 1972). When a cat consumes meat infected with bradyzoite cysts of the parasite, the cyst wall protects the parasites from stomach acid and allows the bradyzoites to emerge in the intestinal tract, where they can infect and proliferate within the gut epithelia. In naïve cats, which encounter *T. gondii* for the first time, the ingested haploid asexual bradyzoites differentiate into micro- and macro-gametes, which then mate to form diploid oocysts (Ferguson, 2002). These oocysts are eventually shed into the environment where they undergo meiosis to form haploid sporozoites.

The oocysts can exist in the environment for a long period and upon being orally consumed by a potential host (by way of food and water contamination) the sporozoites differentiate into asexual tachyzoites, which are fast growing and virulent forms of the parasite. Subsequently, in response to immune attack from the host or other types of stress, the tachyzoites differentiate into slow growing bradyzoites and become tissue dwelling cysts. Since these cysts can persist in the host for a long - in many cases for the entire life of the host– their presence indicates chronically infected hosts.

In cases where the mammalian host happens to be a pregnant female, vertical transmission of tachyzoite stage *T. gondii* from mother to progeny is a common occurrence (Langer, 1963). After the host mounts an immune response to combat acute infection, tachyzoite stage parasites convert into slow-growing bradyzoites, which persist in the host resulting in chronic infection. This differentiation completes the entire life cycle of the parasite (**Figure-1.1**). In host animals, the tissue cysts (containing bradyzoites) are formed in

immune-privileged tissues such as brain and muscle, and bradyzoites can convert into tachyzoites spontaneously (Frenkel and Escajadillo, 1987), which then disseminate to other locations within the host animal or convert back into bradyzoites. When bradyzoites enter the host gut via oral infection; they rapidly convert to tachyzoites, invade the gut epithelia and subsequently disseminate throughout the host animal (Odaert, 1996).

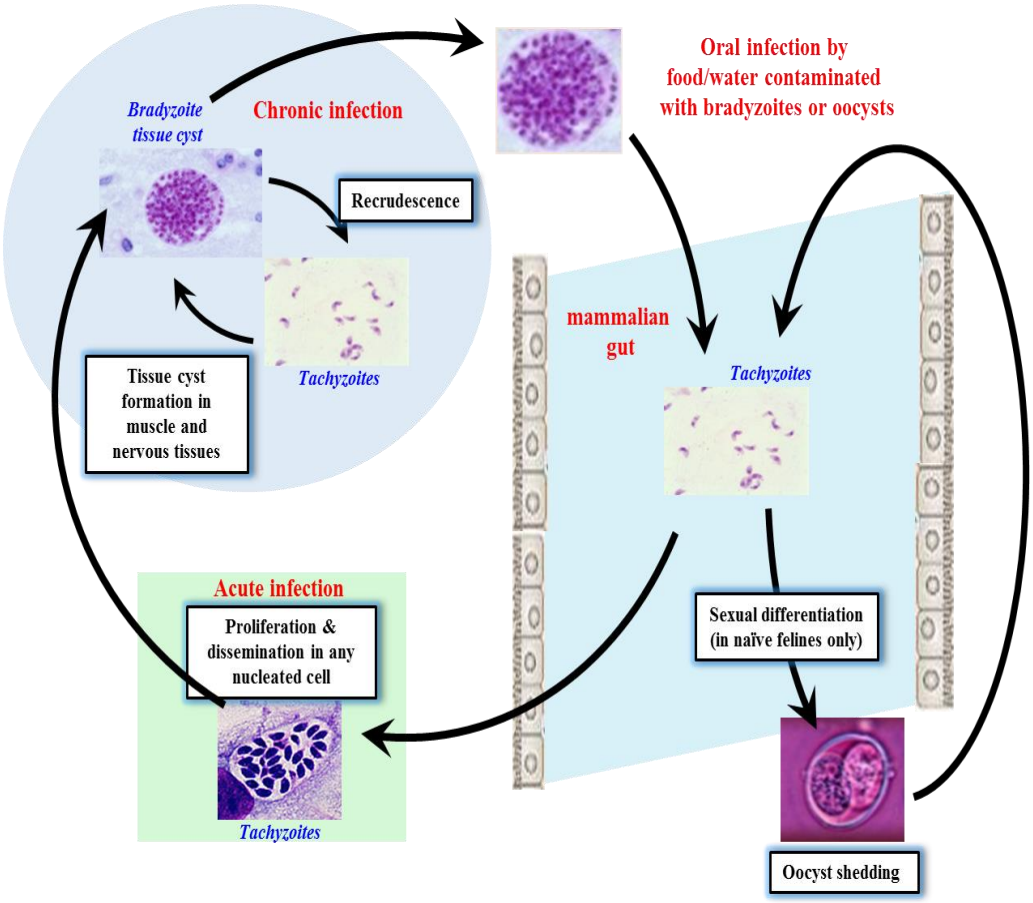

Figure-1.1: **Schematic representation of *T. gondii* life cycle**. Acute infection of host begins with tachyzoite proliferation at the site of infection (usually gut) and dissemination to other parts of the host. Chronic infection is established when tissue cysts containing bradyzoites are formed in muscle and nervous tissues. Consumption of infected flesh by warm-blooded carnivores will result in the initiation of a new cycle of infection. Sexual differentiation takes place if the host happens to be a naïve felid. Images courtesy: Wikipedia; www.nicd.ac.za; www.pharmacies-online.com; Prof. John Boothroyd lab web page (UCSF, USA).

Cellular architecture of T. gondii

The most aggressive and actively dividing form of *T. gondii* is the tachyzoite stage, which are crescent shaped and of roughly 2µM by 5-7µM in size. Being an eukaryotic cell, it possess various membrane bound organelles (**Figure-1.2**) such as the nucleus, a single mitochondrion, endoplasmic reticulum, Golgi body and a distinct four membrane organelle known as the apicoplast, which is a non-photosynthetic plastid harboring important metabolic functions that are considered to be drug targets (Fichera and Roos, 1997). In addition to these there are also specialized set of organelles at the apical end of the cell called rhoptries and micronemes, which are characteristic of all apicomplexan parasites (and hence the phylum name) and which have important role in host cell invasion and egress by the parasite.

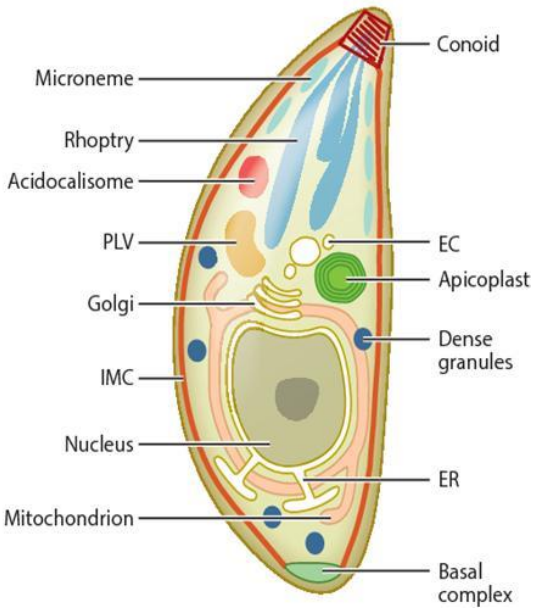

Figure-1.2: Cellular architecture of *T. gondii*. Image adapted from Blader *et al* 2015; *annual rev micro*.69

T. gondii as a model for identifying novel antiparasitic compounds

Currently, only a few drugs are available for clinical use against toxoplasmosis and malaria. However, since malaria is a major health concern, in terms of the number of annual

worldwide fatalities, it has garnered more attention in drug discovery efforts. Despite these efforts, identifying and approving novel antimalarial drugs is a long drawn and costly process, and has been largely ignored by major pharmaceutical companies. However, recent efforts from academia and Medicines for Malaria Venture, Geneva, have greatly expedited this process, and currently a healthy number of compounds are advancing through the Antimalarial drug discovery pipeline (*mmv.org*). However major challenges still remain. The malaria parasite is known to develop drug resistance very quickly and has already developed resistance to all frontline antimalarial drugs, for example chloroquine and artemisinin resistance (Carlton *et al*., 2008; Fidock *et al*., 2008).

Therefore it is conceivable that the malaria parasite will become resistant to any new drugs also. Thus, there is a need for development of mechanistically distinct novel and potent inhibitors, which can be used in combination with other antimalarial drugs. Clinically effective drugs available for treating toxoplasmosis are only a few (example, sulphadoxine/pyrimethamine combination and clindamycin). Identifying effective drugs against toxoplasmosis is difficult due to the chronic nature of the disease and the ability of the parasite to form tissue cysts. Even though currently drug resistance is not a major issue in case of toxoplasmosis, it exists and thus needs to be considered seriously.

For any new antiparasitic drug to be effective, they must target essential pathways and functions in the parasite. Various promising drug targets, including apicoplast and mitochondrial housekeeping and metabolic functions have been identified, and their inhibitors are being pursued as lead molecules (Fichera and Roos, 1997). The availability of high quality functional genomics datasets from *T. gondii* and *P. falciparum* have greatly facilitates the search for novel drug targets in these as well as other apicomplexan parasites. Moreover, experimental tools and techniques required for identifying novel drug targets, such as genetic manipulation, are well characterized and developed in these parasites, which is a

great advantage. Comparative genomics methods and the presence of orthologous proteins performing similar functions in these two parasites has made it possible to directly correlate experimental findings, including those related to drug discovery, between these two parasites. Some pathways or functions identified as drug targets for validation of novel antimalarial compounds are listed in **Table-1**.

Targets	Identity of pathways involved
Apicoplast Metabolism	Isoprene biosynthesis Lipid metabolism Protein synthesis and transcription
Mitochondrial enzymes	Dihydroorotate dehydrogenase Electron transport system
Epigenetic control	Histone deacetylase and acetyltransferase
Protein kinases	Cyclin-dependent protein kinases
Proteases	Cysteine proteases
Transporters	ATPases

Table-1: **Overview of drug targets and their involvement in parasite metabolism**.

In addition to advantages listed above in using *T. gondii* as a model organism for antiparasitic drug discovery, other key factors are the ease with which the parasites can be grown in the laboratory and examined for phenotypic changes by microscopy. Especially in case of studies on organellar morphology and dynamics, *T. gondii* is the preferred organism. In this work (as elaborated in later sections) we have particularly used phenotypes associated with parasite egress from host cells and apicoplast segregation between daughter cells to identify mechanistically novel antiparasitic compounds that are effective against both *T. gondii* and *P. falciparum.*

Genetic methods to study gene function and essential in *T. gondii*

Genetic manipulation has greatly facilitated discovering gene functions that are essential in all organisms – from prokaryotes to eukaryotes. These studies can be targeted, i.e., studying one or a few genes at a time, or genome wide. One way to identify the function

of a gene in its natural setting, i.e., within the cell, is to generate mutations in the gene that result in loss of expression or function and then observe and quantify the associated phenotypic effects. Alternatively, genome wide random mutagenesis can be used to identify gene responsible for specific phenotypic changes or associated with specific pathways and functions. Genetic studies can be broadly classified into as the following two categories.

Forward genetics: Forward genetic approaches are essentially gene discovery experiments in which phenotypic effects resulting from unknown mutations in the genome are first identified and then the complete genetic difference in comparison to the wild type are mapped out before finally identifying the gene(s) responsible. This approach can be very useful to discover novel genetic interaction at the genomic level. More importantly, this can be a reasonably quick approach to score for mutations that are well tolerated by cells (at least in laboratory conditions), and thereby provide valuable information regarding essentiality of genes. In case of *T. gondii*, N-ethyl-N-nitrosourea (ENU) mediated random mutagenesis has been a preferred method for isolating novel mutant strains of the parasite with specific phenotypic effects (Farrell *et al*., 2014).

Reverse genetics: Reverse genetic experiments are done to ascertain the phenotypic effects resulting from loss of expression or function of one or more genes in a targeted manner. This typically involves mutating the target gene(s) and then testing the mutants for various phenotypic effects. This is a very useful method to study the study ortholog functions. For example, if a gene is orthologous in *T. gondii* and *P. falciparum*, studying loss of function effects in the genetically more amenable *T. gondii* can be useful for its functional characterization in *P. falciparum*. In the absence of RNAi pathway in apicomplexan parasites, it was not possible to carryout genome wide screens, but the advent of the CRISPR/Cas9 mediated genome editing technique has dramatically facilitated genome wide

genetic screens in *T. gondii* at least (Sidik *et al*., 2016). A schematic representation of these two genetic approaches is shown in **Figure-1.3**.

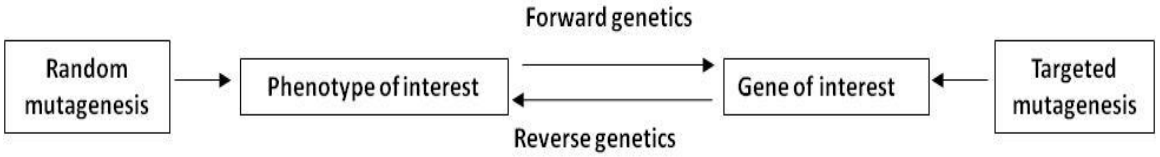

Figure-1.3: **Schematic representation of forward and reverse genetic experiments.**

Experimental methods for the genetic manipulation of *T. gondii* are available and each method is successful in few cases with some inherent shortcomings. Random mutagenesis assays using whole cell, either by chemical or genetic approaches were the only available tools to identify gene specific functions, based on phenotypic effects. Exposure of parasites to mutagens such as ENU and further studying the mutant phenotypes facilitated forward genetic analysis *T. gondii*. Elucidation of nucleotide biosynthetic pathway in *T. gondii* was one of the initial milestones in forward genetic analysis using chemical mutagens (Pfefferkorn *et al.,* 1976). Reverse genetic approaches have become possible and increasingly the method of choice in recent times, as they enable introduction of foreign or reporter genes in parasites along with selectable markers. Genetic manipulation techniques have evolved during the past decade and newer approaches (as described in the section below) are being developed to study apicomplexan parasites.

Tools available for genetic manipulation of *T. gondii*

Availability of transgenic expression systems for constitutive as well as stage specific expression (for example, using *α-tubulin* or *Bag2* promoters, respectively) of gene of interest in *T. gondii*, have made it possible to generate genetically modified lines - either transient or stable - for protein expression and localization, and for phenotypic studies (Soldati *et al*.,

1993). Subcellular localization studies of various proteins of interest can be done in tachyzoite stage *T. gondii* by expressing proteins tagged with fluorescent and epitope tags. At times, protein over expression or the presence of the tag can cause protein mis-localization in extreme cases this can be toxic, resulting in the death of parasite. These unintended outcomes can be largely avoided by expressing the C-terminal tagged version of the protein from the endogenous loci itself, using the native promoter, so that expression levels of the gene is not altered. Such approaches are found to be more suitable for maintaining the normal cellular expression and subcellular localization of any protein.

In contrast to transient expression experiments, where the gene of interest is expressed from a simple expression cassette in a plasmid, for stable gene expression and for manipulating target genomic loci, the plasmid constructs are typically made to facilitate homologous recombination mediated integration of the DNA fragment of interest at the target genomic loci. Wild type *T. gondii* is known to have high non-homologous recombination activity, which promotes random integration of the plasmid construct at various locations within the genome, in response to a strong selection for survival, for example resistance to the drug pyrimethamine mediated by expressing a mutant version of the dihydrofolate reductase thymidylate synthase (*dhfr-ts*) gene. In order to increase the frequency of targeted integration of the DNA fragment of interest in parasite genome, a mutant *T. gondii* strain lacking the KU80 protein (Δ*ku80*), which facilitates non-homologous recombination activity, was engineered, and the Δ*ku80* strain was found to have > 90% efficiency in homologous recombination frequency (Fox *et al*., 2009, Huynh *et al*., 2009).In *Plasmodium sp.*, genome integration occurs exclusively by homologous recombination hence site-specific transposable elements are more commonly used to manipulate genomic loci (Sakamoto *et al* ., 2005).

A handful of selection markers are available for genetic studies in parasites; these include the genes coding for the following proteins: DHFR-TS (Donald *et al*., 1993),

chloramphenicol acetyl transferase (CAT) (Kim *et al*., 1993), bleomycin resistance protein (BLE) (Messina *et al*., 1995), hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) (Donald *et al*., 1996, 1998) and uracil phosphoribosyl transferase (UPRT) (Donald *et al*., 1995). These selectable marker cassettes can be used in random integration as well as homologous recombination experiments. Few of these markers could be used as dual markers or can be used for positive as well as negative selection for transformants. List of these markers along with the inhibitor used for selection is given in the **Table-2.**

Parasite	Markers	Selection confers resistant to	Selection mode	Selection condition	References
T. gondii	HXGPRT	Mycophenolic acid	Positive	MPA (20µg/ml)+Xanthine (50µg/ml)	Donald *et al*., 1996, 1998
T. gondii	CAT	Chloramphenicol	Positive	20µM CML	Kim *et al*., 1993
T. gondii/ P. falciparum	DHFR-TS	Pyrimethamine	Positive	1µM Pyr	Fidock *et al*., 1997
P. falciparum	BSD	Blasticidine	Positive	-	Mamoun *et al*., 1999
P. falciparum	NEO	G418	Positive	-	Mamoun *et al*., 1999
P. falciparum	DHOD	Atovaquone	Positive	-	Painter *et al*., 2007
T. gondii	BLE	Phleomycin	Positive	5ug/ml	Messina *et a*l., 1995
T. gondii	UPRT	5'-fluo-2'-deoxyuridine (FUDR)	Negative	5uM FUDR	Donald *et al*., 1995
T. gondii	HXGPRT	6-thioxanthine	Negative	80ug/ml 6-TX	Donald *et al*., 1996, 1998
T. gondii	YFP	-	Negative	-	
P. falciparum	CD	5-fluorocytosine	Negative	40uM FLUC	Maier *et al*., 2006

Table-2: **List of markers used for transgenic parasite selection in apicomlexan parasites.**

Regulated gene expression is a very useful technique, particularly to study the function and phenotype of essential genes, where the genes cannot be knocked out. A few regulated gene expression systems are available for use in *T. gondii* and *P. falciparum* as discussed below. Tetracycline inducible or repressible systems were the first to be developed for these parasites (Meissner *et al*., 2001). A major limitation of this technique is the need to propagate parasites in the presence of tetracycline, which can be cytotoxic and affect the

wellbeing of the plastid organelle, resulting in delayed death of parasites. Hence, anhydrotetracycline, a relatively non-toxic version of tetracyclin, is used, but the toxicity of this molecule can vary even between different strains of *T. gondii*. This system has been engineered to function effectively using the tetracycline transactivator (TATi) mediated based inducible system in *T. gondii*. TATi is a fusion between the tetracyclin repressor (TetR) and the activating domain of Herpes simplex VP16 protein (Meissner *et al*., 2001). This system has its flaws and has even failed in few cases because of lower activity of VP16 activating domain with parasite transcription machinery. Subsequently, the development of a TATi strain in *T. gondii* has been reported, which currently is the method of choice for conditional disruption of essential genes in *T. gondii* (Meissner *et al*., 2002).

RNAi is a widely used method for gene silencing in many eukaryotic systems but in apicompexan parasites it does not work as the pathway is totally absent (as in *P. falciparum*) or only partially present (as in *T. gondii*; Militello *et al*., 2008). The hammerhead ribozyme, which can be regulated using toyocamycin is also not useful in case of apicomplexan parasites due to toxicity of the molecule (Yen *et al*., 2006, Agop-Nersesian *et al*., 2008). However, the *glm*S ribozyme present in several bacteria, which can be regulated by the non-toxic molecule glucosamine, has been recently shown to mediate relatively tight gene regulation in *P. falciparum* (Prommana *et al*., 2013). Unfortunately, for unknown reasons the *glm*S ribozyme does not work in *T. gondii* (DS Lab data). The protein destabilization domain(the FK506-binding protein degradation domain or DD) has been shown to regulate protein levels within the cells, by targeting the tagged protein for proteosomal degradation in the absence of the rapamycin derived stabilizer molecule called shield-1 (shld-1) (Banaszynski *et al*., 2006, Herm-Götz *et al*., 2007). Although, this method of regulated protein expression works well in case of cytosolic proteins, it is not very effective for

membrane proteins, secreted proteins and proteins associated with organelles (Santos *et al*., 2010).

While genetic manipulation is a robust and powerful way to study gene function and essentiality, they have some limitations as listed below -

- The methods and tools used for genetic manipulation are parasite specific, and for efficient gene manipulation these need to be developed independently for each organism.
- In case of *P. falciparum*, the AT rich genome is not readily accessible for genetic manipulation (Aravind *et al*., 2003).
- Genetic studies on essential genes are extremely difficult to perform in haploid stages of parasite that are easily cultured in the laboratory.
- Gene essentiality can vary in different developmental stages of the parasite and thus needs to be evaluated separately. In case of apicomplexan parasites only certain forms of the parasite can be grown in the laboratory and this makes genetic validation difficult in all life cycle stages.
- Genetic approaches are not well suited to study dynamic cell biological processes that occur on a very short time scale, especially in case of essential genes (Behnke *et al*., 2010).
- Not possible to reverse the phenotypic effect mediated by genetic changes unless a suitable regulated expression system is available for the gene or protein of interest.

Pharmacological studies on apicomplexan parasites

There are two approaches for conducting pharmacological studies– either by target specific inhibitory studies (i.e., biochemical assays using purified proteins to identify inhibitors) or by whole cell screens (i.e., treating live cells with inhibitors and tracking their

viability). Target based screens, although widely pursued at one point, have failed to yield good antiparasitic molecules. This is primarily due to the fact that *in vitro* efficacy against isolated proteins translates very poorly into *in vivo* (cellular level) efficacy. Moreover, toxicity problems will have to be separately addressed. Therefore, whole cell screens are currently the method of choice used for identifying antiparasitic molecules from a panel of small molecule inhibitors. In whole cell assays, the most commonly observed phenotype in an end point assay is the viability of the parasite, i.e., arrest of cell growth and proliferation, in the presence of the inhibitor. However, other more specialized phenotypic assays, specific to the biology of the parasite, such as identifying invasion and/or motility inhibitors, is also conducted using customized phenotypic screens. To address the mechanism of action of the inhibitors in whole cell screens, a suitable assay is required to monitor the specific phenotypic effects pertaining to the likely target or pathway being inhibited by the parasite (example, motility assays). Chemical hits (i.e., compounds capable of completely arresting parasite growth with high potency) identified from the phenotypic screens are potential candidate lead molecules for anti-parasitic drug discovery.

Various different whole cell screens have been developed to date for *T. gondii* and *P. falciparum*. These screens differ in their readout methods and the phenotype being monitored. A list of whole cell screening methods available for both *T. gondii* and *P. falciparum* are listed below.

***Whole cell screens developed for identifying inhibitors of P. falciparum*:**

- The first standardized parasite proliferation assay was developed based on radioactive labeling of nucleic acids using the tritiated nucleotide precursor hypoxanthine. The

extent of radioactive incorporation into parasite DNA is indicative of parasite proliferation.

- Enzyme-linked immune-sorbent assays (ELISA) were developed specifically against parasite antigens like lactate dehydrogenase (LDH), histidine rich protein 2 (HRP2) and heat shock protein 70 (HSP70).The extent to which these proteins are present in the culture is indicative of parasite proliferation. The ELISA assays have been adapted to micro-well format.
- Current method of choice for assessing parasite growth during intra-erythrocytic growth is by staining the parasite DNA with fluorescent dyes such as Hoechst, DAPI or SYBR green I.
- A transgenic parasite line expressing the luciferase reporter has been developed for monitoring the differentiation of blood stage *P. falciparum* into gametocytes. This assay is useful for identifying the so called "transmission blockers", since inhibition of gametocytogenesis is important for cutting off malaria parasite transmission *via* mosquitoes. **Table-3** provides the details of the methods used for inhibitor screening against *P. falciparum*.

***Whole cell screens developed for identifying inhibitors of T. gondii*:**

- A simple assay developed for examining parasite proliferation is by monitoring disruption of the host cell monolayer. Also known as the plaque formation assay, the readout involves staining the infected host cell monolayer after methanol fixation with crystal violet to visualize the zone of clearance resulting from multiple host cell lysis following several rounds of parasite invasion, replication and egress.

- Incorporation of radiolabelled H^3 uracil by the activity of parasite specific uridine phosphoribosyl transferase (UPRT) is used in this assay for determining parasite growth.

Method	Stage	Monitoring	Readout	References
Metabolic labeling	Intra-erythrocytic	DNA synthesis	Scintillation counts	Desjardins *et al*., 1979; Iber *et al*., 1975
DNA staining	Intra-erythrocytic	DNA synthesis	Fluorescence	Smilkstein *et al*., 2004; Bacon *et al*., 2007; Baniecki *et al*., 2007
ELISA	Intra-erythrocytic	Parasite antibody	Absorbance	Kaddouri *et al*., 2006; Noedl *et al*., 2005
Reporter assay	Intra-erythrocytic& Gametocyte	Luciferase expression	Luminescence	Lucantoni *et al*., 2013

Table-3: Available methods for screening small molecule inhibitors against *P. falciparum*.

- Using specific antibodies against the surface antigens of the parasite, ELISA has been developed to measure parasite growth. In this assay the abundance of antigen present is indicative of the extent of parasite growth.
- Flow cytometry based techniques have been developed to monitor the number of host cells infected with *T. gondii* parasites by staining for parasite surface antigen (SAG) which is secreted into the parasitophorus vacuole present within the host cell. Further, transgenic parasite expressing fluorescent marker proteins have been used for dual or multi-color flow-cytometry assays without the need for additional staining steps.
- Transgenic parasites expressing reporter genes like β-galactosidase or firefly luciferase are now widely used in whole cell inhibitor screens. **Table-4** provides the details of the methods used for inhibitor screening against *T. gondii*.

Method	Stage	Monitoring	Readout	References
Monolayer disruption	Tachyzoites	Host cell disruption	Crystal violet staining	Roos *et al*., 1994
Radioactive	Tachyzoites	DNA synthesis	Scintillation counting	Pfefferkorn *et al*., 1976,Nare *et al*., 2002
ELISA	Tachyzoites	Parasite antibody	Absorbance	Merli *et al*., 1985, Derouin *et al*., 1988
Flow cytometry	Tachyzoites	Antibodies or YFP	FACS	Gay-Andrieu *et al*., 1999, Grimwood*et al*., 1996
Fluorescence	Tachyzoites	YFP expression	Fluorescent plate reader	Gubbels *et al*., 2003
Lac Z	Tachyzoites	B-galactosidase	Absorbance	McFadden *et al*., 1997, Seeber *et al*., 1996
Luciferase	Tachyzoites	Luciferase	Bioluminescence	Matrajt *et al*., 2002, Saeij *et al*., 2005

Table-4: Available methods for screening small molecule inhibitors against *T. gondii*.

As stated above, whole cell assays are the method of choice for identifying antiparasitic compounds. The methods described have some advantages and disadvantages, which are listed below.

- When inhibitor treatment induces phenotypic changes in the parasite, it is a good indicator that the small molecule can access intracellular compartments and interact with its target.
- Chemical inhibitors usually work very rapidly, and dosage variations can be used to tease out specific phenotypes (e.g., killing at higher dose but reduced growth at lower dose).
- Chemical inhibitors can be tested against any stage of the parasite, both in culture and in animal models.
- Unlike in case of genetic screens, specific parasite lines (i.e., transgenic lines) are not needed for testing chemical inhibitor effects. This facilitates testing of chemical

inhibitors on any parasite line. In fact, even a completely different species can be tested to evaluate inhibitor activity.

- Conserved phenotypic response to chemical inhibitor treatment across species can help in mechanistic and target identification studies.
- Small molecule inhibitors cannot target ever gene in the genome. Only a portion of the genome (i.e., the druggable genome) is responsive to inhibitor treatment. Therefore, genome wide studies cannot be done with chemical inhibitors.
- Target specificity for small molecule inhibitors is not always guaranteed. It is entirely possible that phenotypic effects can occur due to the inhibition of multiple targets or non-specific targets by the inhibitor. This especially happens at higher doses of the inhibitor and is referred to as "off-target" effects.
- Target identification for novel inhibitors can be difficult, unless it is possible to raise resistant mutants. However, since targets tend to be conserved across species, mechanistic studies in one species can applicable to another species.

It is now fairly common that both genetic and chemical approaches are being used to complement each other in antiparasitic drug discovery studies (**Figure-1.4**). Chemical screening alone cannot address drug mechanism of action, and hence genetic approaches are needed. Small molecules could act either as agonists or antagonists of target cellular processes resulting in distinct phenotypic changes. Similarities between the phenotypic effects elicited by chemical inhibitors and genetic changes can facilitate mechanistic studies and target identification. However, too often, genetic phenotypes can be very drastic (example, cell death) and therefore studying molecular changes in the cell, such as altered gene expression, during chemical treatment, can be quiet useful to delineate the mechanism of action.

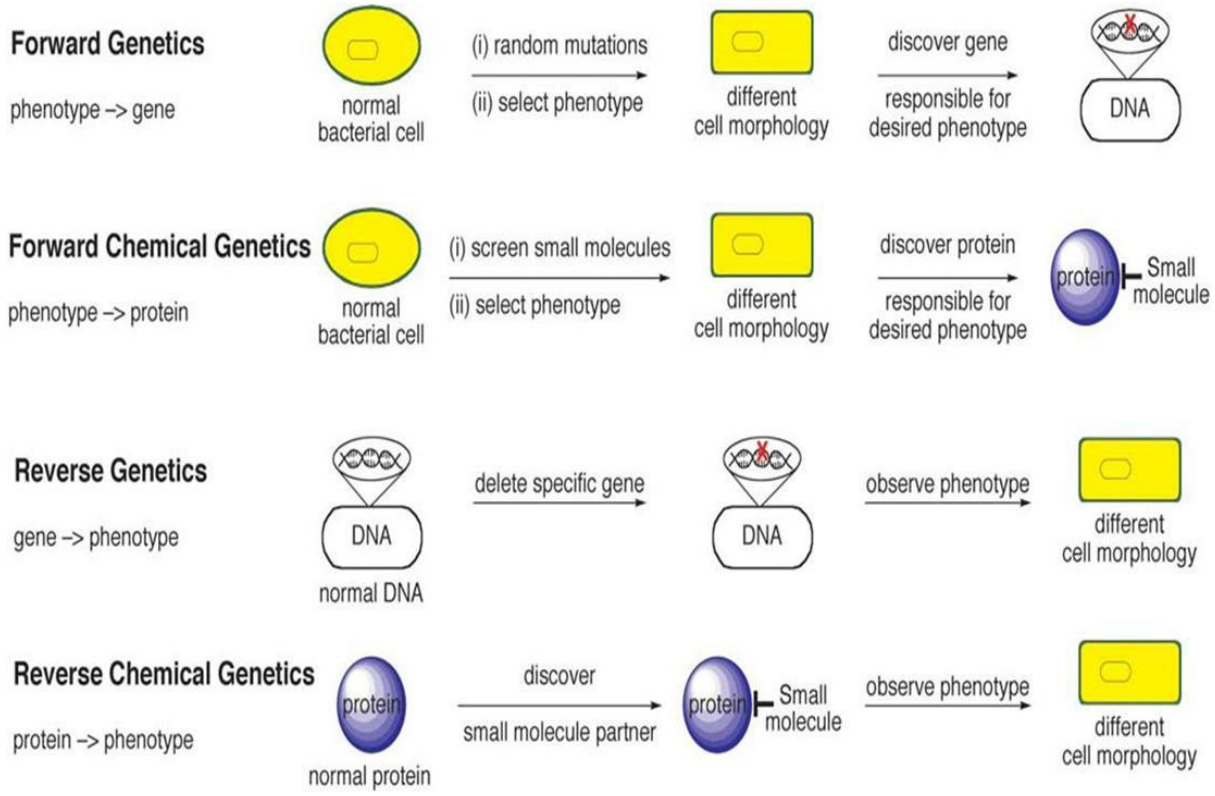

Figure- 1.4: Comparing genetic and chemical genetics approaches. Ref: Chem. Soc. Rev., 2005.

Requirements for carrying out cell based chemical phenotypic screen

Small molecule collections: The success of any chemical phenotypic screen is very much dependent on the quality of the small molecule inhibitor library used. The chemical diversity of the library is critical, as it will ensure that mechanistically distinct molecules are selected as hits from the screens. Broadly, two types of small-molecule collections are available for bioactivity studies. These are -

- **Natural products**: These are usually secondary metabolites isolated from plants. Although it is desirable to have the pure form of these natural products, in many instances crude or partially purified extracts are used in the screens. However, in this case, it is likely that the phenotypic effects observed are a result of the combined effect of many molecules. The selection of these natural products is prioritized based on their use in various forms of traditional medicine.

- **Synthetic organic molecules**: Medicinal chemistry programs world over are focused on generating a variety of synthetic molecules to address drug discovery for various diseases. These synthetic molecules are designed to be bioactive, and they can be completely new ones or analogs of known inhibitory compounds and natural products. Synthetic molecules are also amenable to further medicinal chemistry work during hit to lead optimization studies, and this is an advantage over natural products which can be quiet difficult to access synthetically.

Both synthetic chemicals and natural products are available from various sources, including from commercial suppliers (example, the Sigma LOPAC library) and global institution (example, "Malaria Box" and "Pathogen Box" collections from Medicines for Malaria Venture (MMV)). In fact, MMV has fostered academia-industry partnerships for compiling the two libraries and making them freely available for researches interested specifically in antiparasitic drug discovery. Both synthetic compounds and natural products have distinct issues in further medicinal chemistry efforts during hit to lead optimization studies. Synthetic molecules tend to be poorly cell permeable and lacking in good bioactive groups that are less toxic. Hence there is a need to generate many different chemical analogs for optimizing the bioactivity. Natural products, on the other hand, have good cell permeability properties, but bulk availability and purity are major concerns. They are also not easily synthesized or chemically modified in the laboratory. When dealing with extracts, which are a complex mixture of chemicals, identifying the active principle can be challenging.

Assay development for phenotypic screens: Development of a suitable assay for detecting and quantifying the phenotype of interest is a major bottleneck in phenotypic screens. This

also has a major influence on the outcome of the bioactivity screens. The following points are useful as a guide while developing phenotypic screens -

- The screen should be robust and amenable to miniaturization (for 96, 384 and 1536 well formats). It should be compatible with automation
- Liquid handling during the assay should be efficient and accurate
- The assay protocol should mandate the inclusion of controls to account for possible errors (Positive and negative controls, as well as technical and experimental controls)
- The assay should be reproducible and the potency of the selected hit molecules should be consistence.
- Assay validation methods should be available, for example by confirming the bioactivity of known inhibitors.
- For phenotypic screens, a good microscopy or flow cytometry based readout methods must be available
- Finally, when assay readout is based on the expression of reporter proteins, the appropriate transgenic parasite lines must be available.

Nowadays, it is possible to carryout phenotypic screens in a high throughput manner using imaging platforms, which can handle micro plates. This approach is popularly called as high content screening, since the extent of imaging carried out for these assays is very large. In these assays, imaging is done by automated microscope which can image cells grown in multiwall plates. The relatively small size of protozoan parasites requires imaging at high magnification, which in-turn depends on good microscopy facility. The image data generated from such experiments will be analyzed by dedicated software for data compilation and interpretation. In some cases the software can be programmed to look for the phenotype of interest using pattern recognition algorithms or based on differential expression of different

marker proteins. Archival, retrieval and analysis of the data exported out of the software plays an important role in the interpretation of results.

Conclusion

Chemical biology approaches have the potential to accelerate our understanding of apicomplexan parasite biology and facilitate antiparasitic drug discovery. The recent trend is to combine chemical screening approaches with that of genetic screens to address inhibitor-target protein-phenotype relationships. Particularly, these approaches have been successfully developed and implemented for *P. falciparum* and *T. gondii*. Identifying good drug-like compounds that specifically disrupt cellular processes in the parasite is not easy and identification of antiparasitic leads targeting essential proteins in the parasite and lack off-target effects in the host systems is the great challenge in drug discovery process. Advances in screening technology and genetic toolkits have made it possible to score thousands of mutants and millions of inhibitors in high throughput approaches. In this regard, what is currently needed is a collection of robust screening methods, capable of interrogating various parasite specific phenotypes– such as, host invasion, replication and growth, egress, organelle dynamics, metabolic functions, and differentiation. This can be a significant bottleneck, especially in case of parasites. Thus, my work was primarily focused on developing various phenotypic screens and to use them for, tracking parasite specific processes, with the goal of addressing the mechanism of action for novel inhibitors and characterize novel gene functions.

Objectives of the thesis work

The main focus of this thesis work is to identify novel small molecules inhibitors against *T. gondii* and characterize the mechanism of action (MOA) for selected inhibitors. The following are the proposed objectives of the thesis work –

1. To carry out a comprehensive anti-toxoplasma screening using small-molecule inhibitors present in the MMV malaria box, MMV pathogen box, and the Sigma LOPAC library. These screens will be used to identify inhibitors causing immediate as well as delayed death of the parasite.
2. To carry out detailed phenotypic studies with the delayed death inhibitors in order to identify the effect of these molecules on the morphology and segregation dynamics of the apicoplast organelle.
3. To address the mechanism of action (MOA) for selected inhibitors which are found to impart delayed death on the parasite by inducing apicoplast mis-segregation.

A detailed description of the background information, methodology and results for the experiments carried out to address the objectives stated above is discussed in next chapters.

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Chapter 2

Identifying molecules with anti-toxoplasma bioactivity using whole cell assays.

Introduction

As discussed in Chapter-1, *T. gondii* is an excellent model for drug discovery against apicomplexan parasites, especially where MOA studies are needed. New drugs are needed against toxoplamosis, especially to effectively control acute proliferation of parasites following recrudescence in immunocompromised patients and in case of congenital infection. Effective elimination of tissue dwelling latent cysts in chronically infected people is also a necessity. However, since toxoplasmosis is not a killer disease (i.e., fatalities due to this disease are rare and few), concerted drug discovery efforts are lacking. Nevertheless, techniques for cell-based screens for inhibitor identification are established for *T. gondii* and can be carried out with laboratory cultivated parasite strains.

The other important requirement for carrying out anti-toxoplasma screens is the availability of suitable collection of inhibitor molecules. In this study, we have used a total of three different inhibitor collections. These are the “Malaria Box” and “Pathogen Box” collections of antiparasitic molecules, managed and distributed by the Medicines for Malaria Venture (MMV), Geneva. The other chemical collection is the Sigma LOPAC library, which is a compilation of bioactive molecules with known mechanism of action against primarily mammalian targets. Further details on these inhibitor collections, is given in the following sections.

Parallel to this study on anti-toxoplasma activity, antimalarial screens were also carried out in our laboratory for these inhibitors. This provides an opportunity to compare the potency as well as phenotypic effects (when available) between these two pathogens. Due to the close evolutionary relationship between these two parasites, inhibitor effect on conserved cellular processes, such as parasite egress from host cells and metabolic functions in the

mitochondrion and apicoplast, are likely to be mediated *via* inhibition of orthologous proteins. This can facilitate MOA studies and validation in two important human pathogens.

Details of the chemical libraries used in this study

Three different chemical libraries have been used for in the anti-toxoplasma screening assays that will be described in this chapter. These libraries are described below.

***The MMV Malaria Box*:** The Malaria Box collection includes selected 'hit molecules' identified from three different large-scale antimalarial screening carried out by two pharmaceutical companies (Novartis and GlaxoSmithKline (GSK)), and one academic group (St. Jude Children's Research Hospital). A majority of the inhibitors included in this collection have sub-micro molar potency against the human malaria parasite *P. falciparum*. A key bottleneck in translating these hits to lead antimalarial molecules is the lack of mechanistic details on how these molecules act and what their targets are. To expedite mode of action studies on these promising molecules, MMV handpicked 400 different molecules of diverse chemotypes - 200 drug like and 200 probe like molecules - to constitute the 'Malaria Box' collection in 2013.An overview of selection pipeline to include antimalarial compounds into the Malaria Box collection is shown in **Figure-1**.

The MMV Pathogen Box: Following the successful dissemination of the MMV malaria Box to the malaria community and response of researcher in evaluating these molecules, MMV launched another library of inhibitory molecules in 2015 called the MMV Pathogen Box. Similar to the Malaria Box, the Pathogen Box also contains 400 diverse drug like chemotypes, most of which were previously shown to be active against a variety of parasites collectively responsible for different neglected infectious diseases. The targets diseases for these inhibitors occur largely in tropical and subtropical countries, affecting billions of people living in poverty and in contact with animal and insect vectors of these diseases.

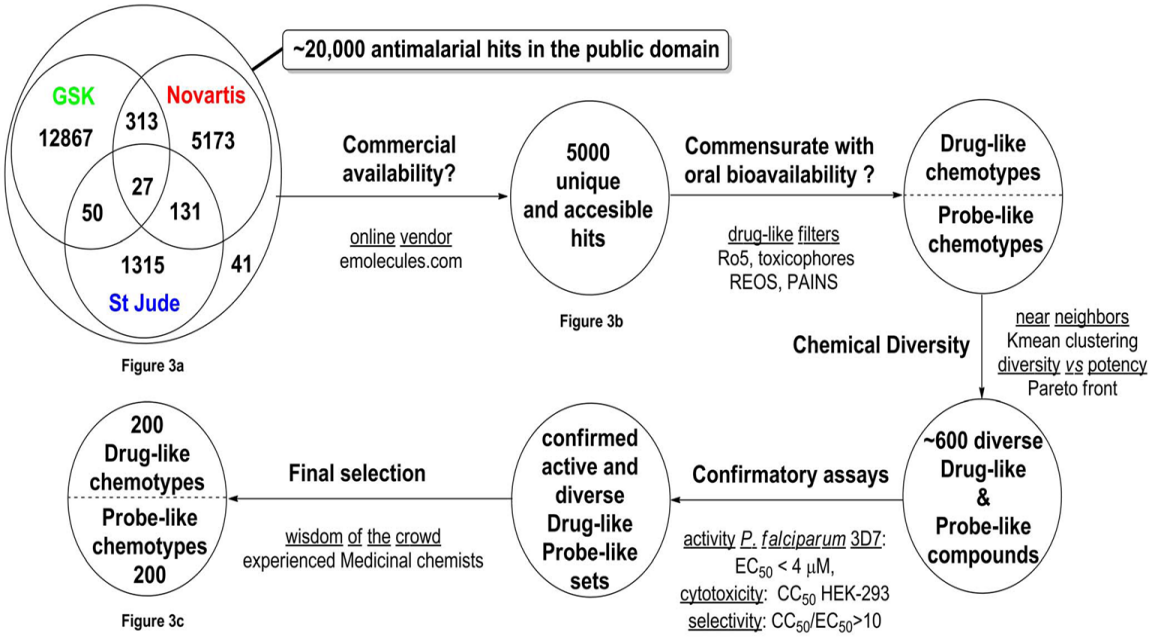

Figure-2.1: Selection pipeline used for compiling the MMV Malaria Box library. Adapted from Spangenberg *et al.*, 2013.

These diseases include ascariasis, buruli ulcer, chagas disease, cryptosporidiosis, hookworm infections, human african trypanosomiasis (sleeping sickness), visceral & cutaneous leishmaniasis, lymphatic filariasis, malaria, onchocerciasis (river blindness), schistosomiasis, trichuriasis and tuberculosis. MMV is currently still distributing this library free of cost to researchers interested in delineating the bioactivity and mechanism of these molecules against these infectious disease pathogens. An overview of selection pipeline used to compile the 400 molecules for inclusion into the Pathogen Box is shown in **Figure-2.2**.

The Sigma LOPAC library: The Sigma Aldrich Company has compiled a set of pharmacologically active small molecules which is known as Sigma Life science's Library of pharmacologically active compounds (LOPAC). This library consists of a collection of 1280 inhibitors, with established mechanisms and known targets in mammalian system.

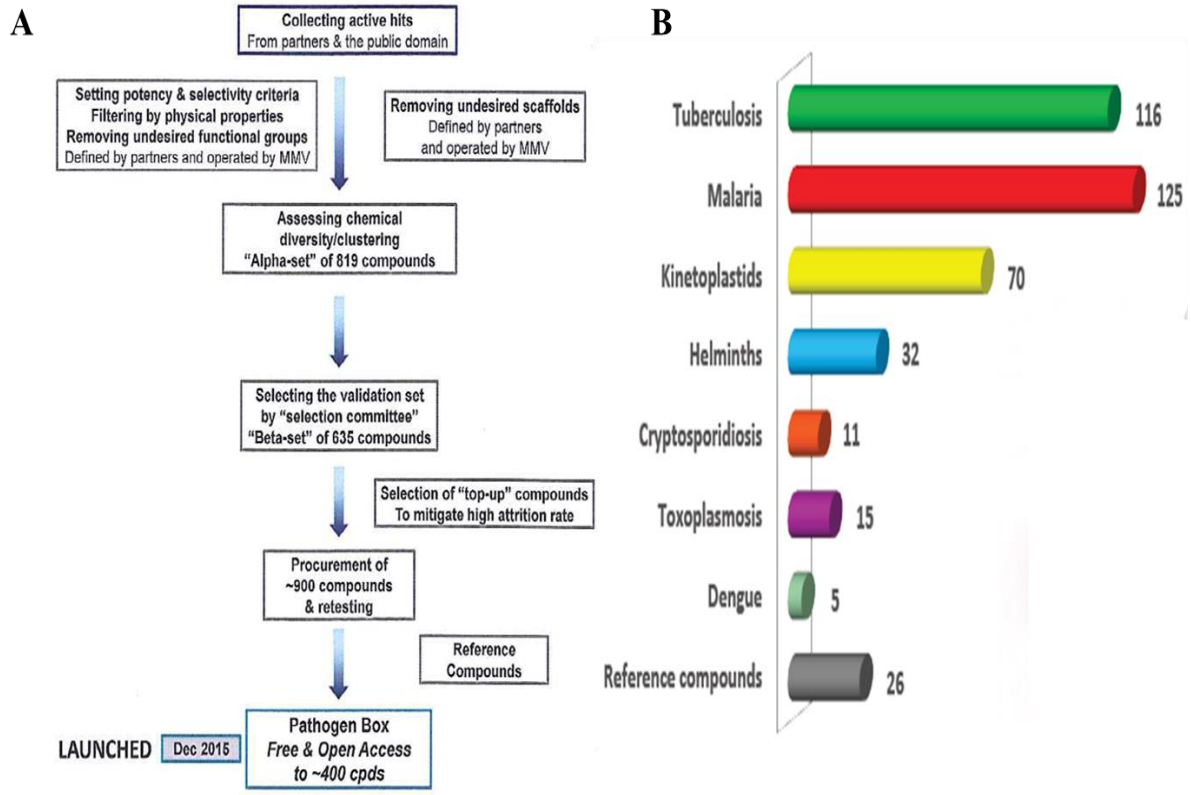

Figure-2.2: A) **Overview of the selection pipeline for assembling the MMV Pathogen Box library**. B).The various infectious diseases against which the Pathogen Box molecules have been tested previously and shown to be active. The number shown above the bars indicate the total number of molecules present in the library that were tested against these diseases.

Detailed pharmacological and clinical datasets are also available for some of LOPAC molecules and covers all major drug target classes, such as GPCRs and kinases. Many of the LOPAC molecules are also currently marketed drugs, with pharmaceutically active chemistry with annotated biological function and hence suitable for drug repurposing applications. An important advantage of the LOPAC library is the availability of pure compounds, which can be purchased from a reliable source, and thus compound availability is not a constraint as is the case for the MMV collections. The overall composition of the LOPAC library in terms of the associated target classes is shown in **Figure-2.3**.

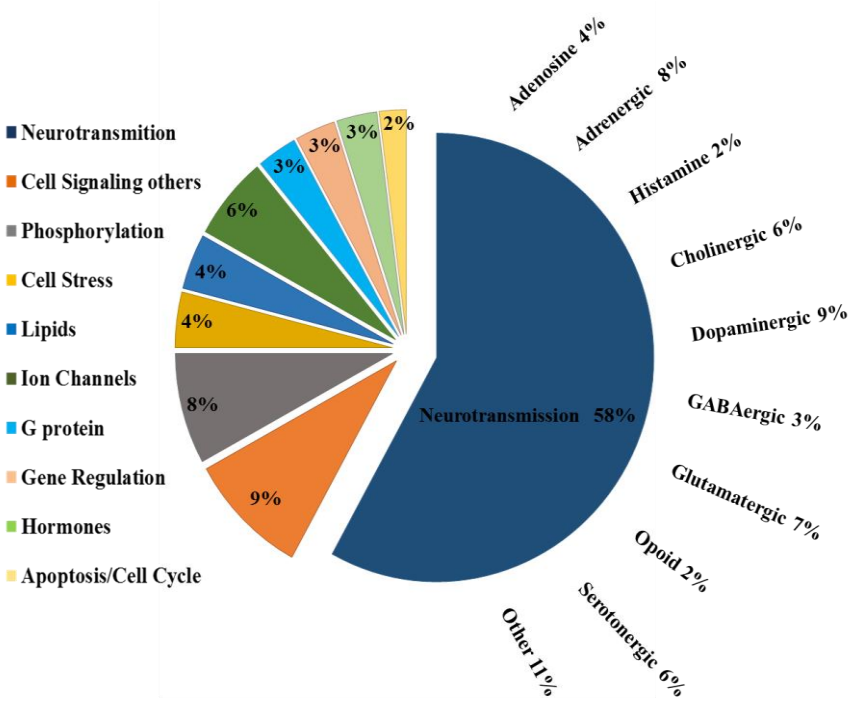

Figure-2.3: **Distribution of LOPAC molecules with respect to their target classes in mammalian system**. Most of the inhibitors are known to act on the neurotransmission pathway, while cell signaling pathways come in second.

Materials and Methods

Culturing tachyzoite stage T. gondii*:* Being an obligate intracellular parasite *T. gondii* (RH Type I strain) requires a host cell to grow and replicate in *in vitro* culture. For all experiments done in this thesis work, the tachyzoite stage parasites, along with the human foreskin fibroblast (HFF) as host cells, were used. The HFF cells were obtained from ATCC and the wild type parasite strains were obtained from the laboratory of Prof. David S. Roos, University of Pennsylvania, Philadelphia, USA. HFF cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM GlutaMAX and 50 µg/ml Gentamicin, in a 37°C humidified incubator maintaining 5% CO_2 (Roos *et al., 1994*). A confluent monolayer of HFF cells was used to infect with a definite number of extracellular tachyzoites depending on the culture vessel and

experiment design. Parasite culture medium was the same as the host cell maintenance media, except that FBS was used at 1%.

To harvest intracellular parasites infected monolayer was scraped using a scraper and this suspension was passed through a 25-gauge needle to lyse the HFF cells and physically release the intracellular tachyzoites. The lysed suspension contained host cell debris and free extracellular tachyzoites, which were further filtered and separated from host cell debris through a membrane having 3 µM pores. The parasite suspension was diluted and counted using a hemocytometer. These purified tachyzoites were used to set up various assays, and details of these assays have been provided in the relevant experimental sections.

Culturing blood stage P. falciparum: *P. falciparum* (3D7 strain) was used to validate some of findings from *T. gondii* screening experiments. Parasites were cultured in 2.5% hematocrit in RPMI-HEPES medium at pH 7.4 supplemented with hypoxanthine 50 µg mL^{-1}, $NaHCO_3$ 25 mM, gentamicin 2.5 µg mL^{-1}, and Albumax II (Gibco) 0.5% wt/vol. To obtain enriched ring stage parasites, standard sorbitol synchronization method was used (Trager and Jensen, 2005).

T. gondii transfection protocol: Freshly isolated tachyzoite stage *T. gondii* were washed and resuspended in complete parasite culture medium. 50 µg of linearized plasmid DNA dissolved in 100 µl of fresh culture medium was mixed with 300 µl of parasite suspension (~$1x10^7$ cells/ml), and the mixture was electroporated using a Gene Pulser Xcell™ electroporation system (Biorad) with capacitance and voltage settings at 10 Faraday and 1.5 kV, respectively. Transfected parasites were then allowed to infect a monolayer of HFF cells and incubated for 12 hours under optimal growth conditions. Infected HFF monolayers were then washed once, and 1µM pyrimethamine was added to culture medium and keep on

changing with fresh medium till we get stable transgenic lines, which were then cloned out by limiting dilution method.

Generation of transgenic parasite lines expressing the firefly luciferase gene: The firefly luciferase gene (PCR amplified from pGL3 plasmid, Promega using Takara LA Taq polymerase) was cloned into a modified pBluescript plasmid backbone as a BglII and NheI fragment for expression under the constitutively active *T. gondii* beta-tubulin promoter, and a *Tg*SAG 3' UTR. This plasmid includes the DHFR-TS expression cassette for selecting stable lines of transfected parasites (RH-Luc). The RH-Luc parasites constitutively express the firefly luciferase gene from a stably integrated plasmid under selection by Pyrimethamine.

Optimization of the inhibitor screening experiments: A two-fold serial dilution, starting with $5x10^3$ parasites was plated in 50µl total volume in a 96 well plate. 50µl of 2x luciferase assay reagent (Promega) was then added and mixed well. The plates were immediately subject to luminescence reading using a VarioSkan Flash plate reader (Thermo Fischer, USA). The samples were tested in triplicates and the raw data was analyzed and plotted using Excel spreadsheet program (Microsoft, USA). Samples were analyzed in triplicates. In order to adapt the assay to 96 well plate formats, we standardized the no of parasites to be inoculated into each well of the plate. First 10^3, $5x10^3$ and 10^4 parasites were inoculated into confluent HFF monolayers grown in 96 well plates in a total culture volume of 200µl per well. After 48 h of growth in optimal conditions, 150µl of culture medium was removed and luciferase assay was carried out as described above.

Identifying inhibitors of tachyzoite stage T. gondii growth: The RH-Luc parasites were used for the primary screening used for hit identification from the chemical libraries. Luminescence measurements were used to determine parasite growth inhibition and estimate the EC_{50} values for the inhibitory molecules. The assays were setup in 96 well plate format,

and 100µl of culture medium containing $5x10^3$ parasites were inoculated into each well having a confluent monolayer of HFF cells, pre-seeded with 100 µl of culture medium containing compounds at either 10µM (for growth inhibition studies) or in serial two fold dilution from 10µM to 0.01µM (for determining EC_{50}). Each plate also included a standard drug as positive control (atovaquone at 1µM) and 1% DMSO treated cultures as negative controls. Inhibitor treatment was done in triplicates for growth inhibition and in duplicates for determining EC_{50}. The controls were setup as four replicates each. After 48 hours of growth in optimal growth conditions, 150 µl of culture medium was removed and 50 µl of 2X luciferase assay reagent (Promega) was added and mixed well before immediately taking reading in a Varioskan Flash (Thermo Fischer, USA). The raw luminescence readings were then processed using Microsoft Excel spreadsheets for calculating percent growth inhibition and EC_{50} estimation.

Estimation the host cell cytotoxicity for inhibitor molecules: Cytotoxicity data for the MMV Box molecules was previously determined and they were found to be non-toxic to various mammalian cell lines. Even though cytotoxicity data for LOPAC may be available, we did not have access to it. Therefore, we have tested cytotoxic effects of LOPAC molecules at 10µM as these are inhibitors of mammalian cell and it is important to show their therapeutic index in favor of antiparasitic activity. Cytotoxicity determination was done using Human foreskin fibroblasts (HFF cells) using a colorimetric assay based on the metabolic conversion of resazurin to resafurin within live cells only (O'Brien *et al*., 2000). Cells were trypsinized, counted and then plated out into 96 well plates (NUNC) at ~$5x10^3$– $8x10^3$ cells /well. These plates were allowed to incubate for 6-7 days to attain confluence and were then used to perform cytotoxicity assays. Cells were treated with 10µM concentrations of all inhibitory molecules in DMEM without FBS in triplicates along with 20% DMSO (toxic concentration) and untreated cells as positive and negative controls. Cells with experimental sets and

controls were incubated for 48h after which resazurin (Sigma Aldrich) (10mM stock in water) is added to each well making it to a final concentration of 100µM. After 12-15 h incubation, the resafurin produced was detected *via* fluorescence measurements using a Thermo Varioskan Flash (Thermo Fischer, USA) plate reader. Excitation and emission wavelengths were set to 560nm and 590nm respectively.

Plaque formation assays with T. gondii: When tachyzoite stage *T. gondii* proliferates rapidly, they destroy the host cells, which they invade and replicate in. When multiple host cells are destroyed in the same vicinity due to parasite proliferation, then in that location, the host cell monolayer is completely cleared, resulting in the formation of small visible plaques, similar to typical viral plaques. Plaque formation by *T. gondii* tachyzoites can take anywhere from 8 - 10 days, and therefore provides a way to quantify long term growth (~70 -100 cycles of cell division).

The plaque forming ability of *T. gondii* tachyzoites was tested in the presence of inhibitors to detect 'delayed' effect of molecules in inhibiting parasite growth. Molecules, which were found to have very little or no inhibitory effect on parasite growth in the primary screens, i.e., those with < 20% growth inhibition at 10µM inhibitor concentration over 48 h were used for testing delayed death effects on parasites. The plaques were initiated by inoculating 50 tachyzoites into each well of a 6 well plate containing host cell monolayer and 10µM of selected compounds pre-seeded into the culture media. In case of the LOPAC molecules, the assays were set in 24 well plates. The plates were left undisturbed for 8-10 days in optimal growth conditions, after which the infected monolayers were fixed with methanol and stained with crystal violet to visualize plaque formation. The plaques were then imaged and all images were processed using Image J software to determine plaque area and count in each well. Area of plaque from inhibitor treated cultures was compared to that of

untreated and 1% DMSO treated controls. 10µM clindamycin (known to cause delayed death of *T. gondii*) and 1µM pyrimethamine or atovaquone (acute killing of parasite) were used a positive controls for parasite killing.

Preparation of stock and working solutions of inhibitor molecules: The MMV molecules were obtained from MMV, Geneva, as stock solution in 96 well plate formats and stored in -80°C until further use. 90 µl of cell culture grade DMSO (Sigma Aldrich, USA) was added directly to the plate supplied by MMV containing 10mM stocks, to make the 1mM working stocks that were eventually used for setting up the various screening assays. The Sigma LOPAC library, as purchased from Sigma Aldrich, was received in 20 different 96 well plates, as 25µl of 10mM stock each. All molecules were diluted further to 1mM in separate plates and this stock was eventually used for setting up various assays. For both *P. falciparum* and *T. gondii*, the top concentration used either in killing or phenotype assays was 10µM. For EC_{50} determination, a twofold serial dilution of the compounds was made, starting at 10µM. Atovaquone (*P. falciparum* and *T. gondii*), chloroquine (*P. falciparum*), clindamycin (*T. gondii*) and chloramphenicol (*T. gondii*) were used as standard positive controls in the killing and phenotypic assays. The concentration of the standard drugs used in the various assays is given in the description of individual assays.

Results

Generation of transgenic line expressing the firefly luciferase gene: Luciferase cDNA (1.6kb) was amplified from pGL3 vector and cloned into the p*tub* vector (used for expression studies in *T. gondii*) as a BglII/NheI fragment (**Figure-2.4**). The modified plasmid was sequenced to confirm the presence of the desired gene fragment.

Optimization of luminescence assay using the RH-Luc parasites: A clonal isolate of the RH-Luc parasites was tested for linearity for luminescence readout and detection, over a twofold serial dilution of the parasite count.

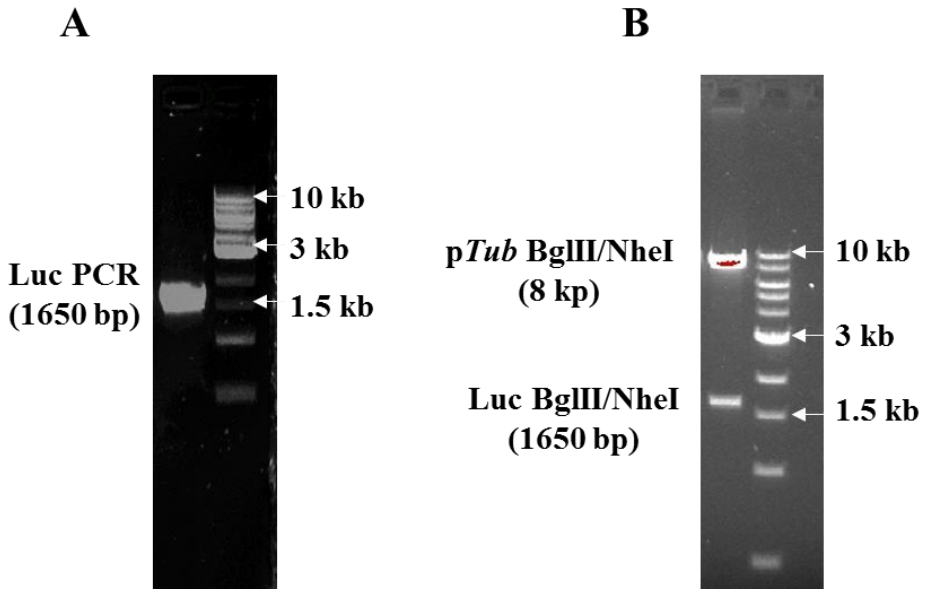

Figure-2.4: **Generation of recombinant plasmid to express luciferase in *T. gondii***. A) Amplification of Firefly Luciferase cDNA. B) Confirmation of final clone of the Firefly Luciferase cDNA in p*tub* plasmid.

The results from the luminescence assays indicate good linearity between parasite count and luminescence read out. We tested the linearity of the luminescence detection technique following inoculation of HFF monolayers in 96 well plates with varying number of parasites and allowing them to proliferate for 48 hours under standard growth conditions. Based on the results obtained from this experiment, we decided to inoculate $5x10^3$ tachyzoites per well for the drug inhibition assays (**Figure-2.5**).

Overview of assay for inhibitor screening in T. gondii* and *P. falciparum: Three inhibitor libraries were screened to identify growth inhibitors of *T. gondii and P. falciparum*. In brief,

total 2070 molecules (1280 LOPAC, 390 Malaria Box and 400 Pathogen Box) were screened in this study to test their effect and potency on parasite growth inhibition.

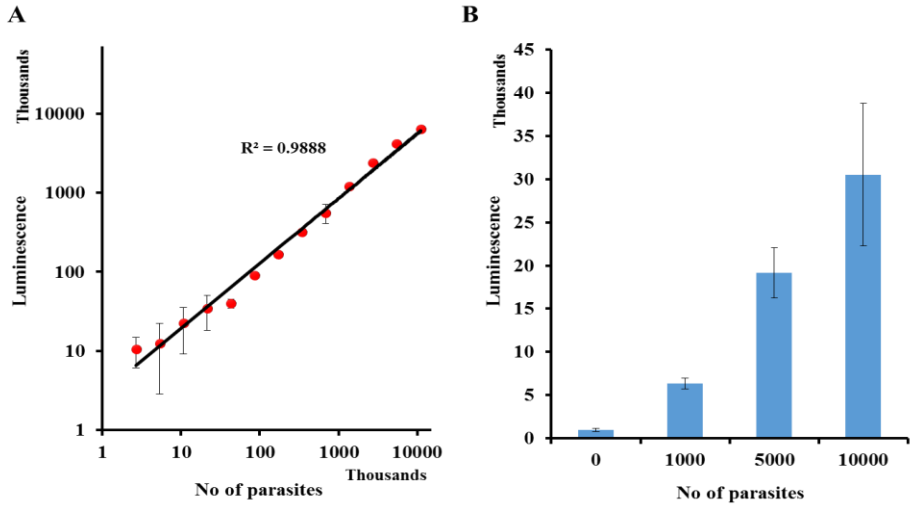

Figure-2.5: **Optimization of luminescence assay parameters for RH-Luc transgenic parasites**. (A) Checking the linearity of luminescence detection using extracellular RH-Luc tachyzoite stage parasites. (B) Detection of luminescence readout following 48 h of proliferation starting with varying number of parasites inoculated into host monolayers.

We reasoned that such an approach will help to identify parasite-specific, as well as cross-species, effects of these molecules during the life cycle stages that are relevant to disease progression and clinical outcome, such as the asexual intraerythrocytic stage of *P. falciparum* and the tachyzoites stage of *T. gondii*. To do this, we undertook a multipronged yet targeted phenotype screening approach, as schematically depicted in **Figure-2.6**. Before embarking on the phenotypic studies, it was important to derive the bioactivity of all compounds in both of these parasites. A summary of the data from these screening experiments is compiled in **Table-5 & 6**.

T. gondii growth inhibition screens against the Sigma LOPAC library: 1280 molecules from Sigma LOPAC library were screened using the luciferase assay. Out of 1280 molecules,

116 molecules showed inhibition of parasite growth by > 80% with respect controls (untreated or 1% DMSO treated parasites). For these molecules we determined the *EC50* values and identified a total of 27 molecules, which inhibited parasite growth with nanomolar efficacy (i.e., EC_{50} < 1µM). A list of these 27 molecules is given in the table in Appendix-I.

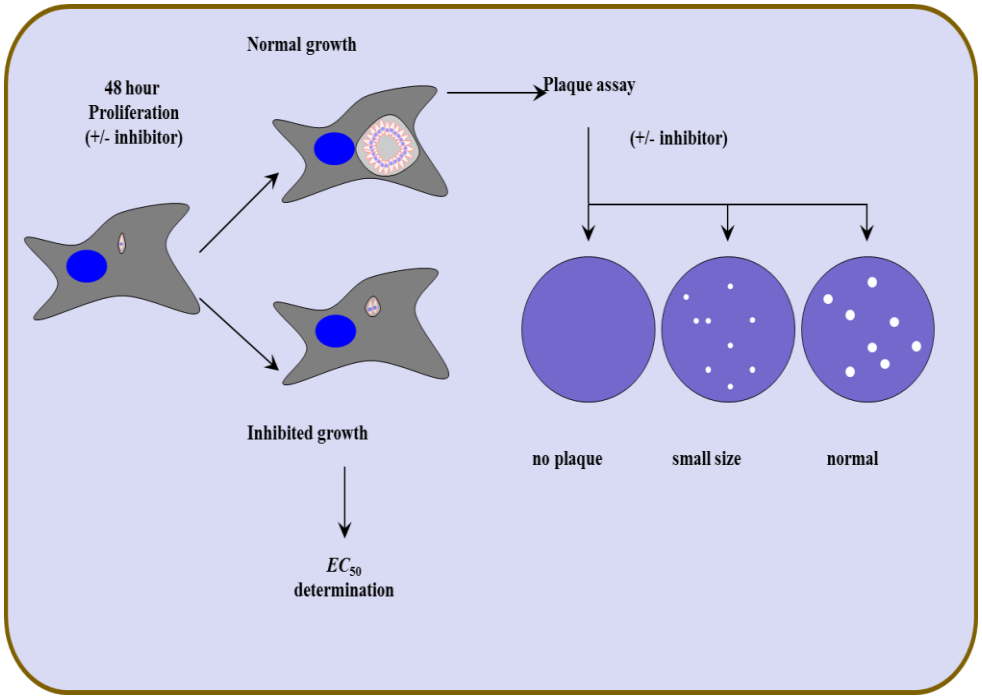

Figure-2.6: **Overview of the primary screening pipeline for the three chemical libraries used in this study**: The left side on the panel depicts the acute growth inhibition assays, which are completed by 48 h, while the right side of the panel shows the plaque assays, which are completed over 8-10 day periods.

Library	Compounds	Active (>80% inhibition)	Inactive (<20% inhibition)	EC50 (<1µM)
Sigma LOPAC	1280	116	849	27
MMV Malaria Box	390	119	134	49
MMV Pathogen Box	400	107	169	45
Total	2070	342	1152	121

Table-5: Summarized results from anti-toxoplasma screening.

Library	Compounds	Active (>80% inhibition)	Inactive (<20% inhibition)	EC50 (<1µM)
Sigma LOPAC	1280	111	461	33
MMV Malaria Box	390	390	0	168
MMV Pathogen Box	400	173	n/d	98
Total	2070	501	461	299

Table-6: Summarized results from anti-malaria screening.

We also identified 849 molecules from the LOPAC library, which had negligible effect on parasite growth, i.e., < 20% inhibition in the primary screening assay (**Figure-2.7**). A parallel effort was taken to screen the LOPAC molecules on intraerythrocytic stages of *P. falciparum*, and identified 111 molecules with > 80% growth inhibition effect, of which 33 molecules had nano molar potency (i.e., EC_{50} < 1µM). We then compared the EC_{50} values for *P. falciparum* and *T. gondii* and identified 7 molecules with nano molar potency against both parasites. Details of these molecules are compiled and shown in **Table-7**.

Host cell cytotoxicity assays on LOPAC molecules*:* Cytotoxicity assay were performed in order to check for inhibitory effects on HFF cells for the 116 LOPAC molecules, which were found to have potent antiparasitic activity. Out of these 116 molecules, 8 showed toxicity towards HFF with the growth inhibition being more than 50% at 10µM concentration. The identity of these molecules are Calmidazolium chloride,' Z-L-Phe chloromethyl ketone, Cantharidic Acid, Ivermectin, Gossypol, Auranofin, Sanguinarine chloride and Thapsigargin. These molecules excluded from any further assays that were carried out with the LOPAC molecules.

T. gondii plaque forming assays with LOPAC molecules*:* As described in the methods sections, those molecules which showed < 20% inhibition of parasite growth at 10µM concentration were selected for testing their effect on the long term growth of *T. gondii* resulting in delayed death of the parasite. From among the LOPAC molecules that were less

potent, plaque assays identified 6 molecules, which induced delayed death of the parasite. The details of these molecules are listed in **Table-8**.

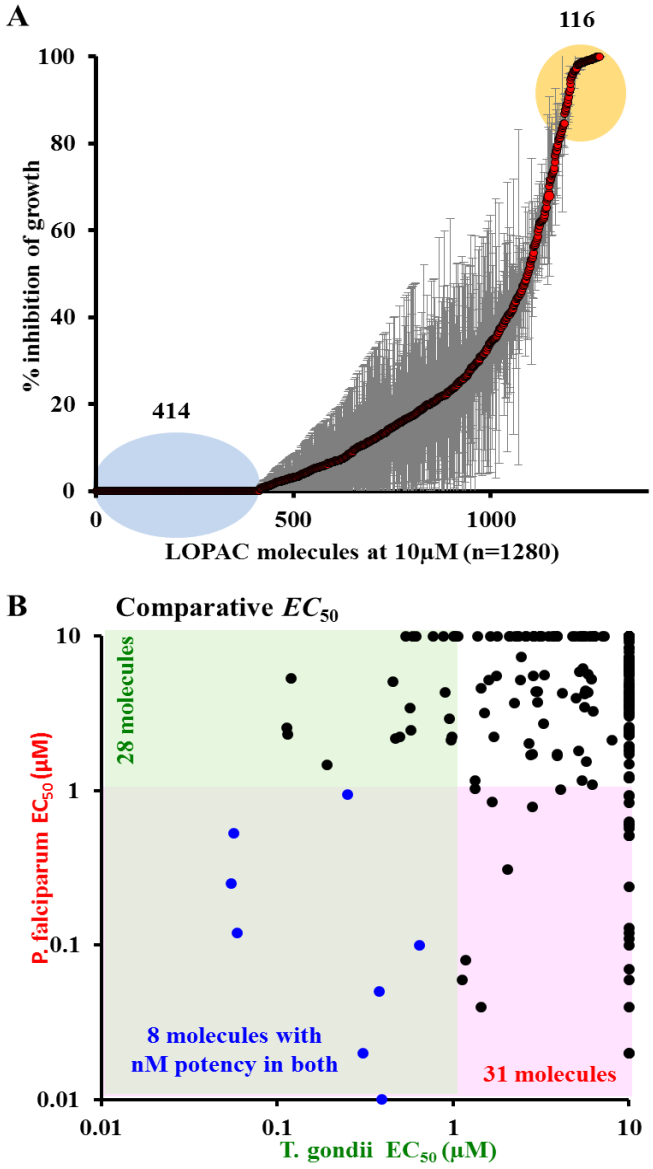

Figure-2.7: Effect of LOPAC molecules on the growth of *T. gondii* and *P. falciparum*. A) *Toxoplasma* growth inhibition assays using a 10µM fixed concentration of LOPAC molecules. The % growth inhibition values are plotted for each molecule after normalizing with control datasets. In this assay the inhibition values obtained for 1µM atovaquone treated parasites was treated as 100% growth inhibition. B) Correlation of the EC_{50} values obtained for the LOPAC molecules against *T. gondii* and *P. falciparum*. The 7 molecules with nM potency against both parasites are highlighted in blue. Molecules whose EC_{50} were not determined (for those with <80% growth inhibition at 10µM) are plotted at 10µM EC_{50} for the respective parasites.

Name	Class	Known mechanism of action	T. gondii EC_{50}(µM)	P. falciparum EC_{50}(µM)
PD-180970	Cell signaling/ tyrosine kinase	PD-180970 is a potent inhibitor of the p210 Bcr-Abl tyrosine kinase.	0.05	0.25
CP-100356 monohydro chloride	Cell Signaling	CP-100356 is a specific inhibitor of MDR1 (P-Gp), the protypical ABC transporter and it also inhibits prazosin transport in human breast cancer resistance protein-transfected MDCKII cells	0.06	0.53
Artemether	Immuno modulators	Artemether is a methyl ether derivative of artemisinin. It is used against multi-drug resistant strains of falciparum malaria.	0.31	0.02
Dequalinium chloride hydrate	K+ Channel	Selective blocker of apamin-sensitive K+ channels	0.39	0.01
Diphenyl eneiodonium chloride	Nitric Oxide	Endothelial nitric oxide synthase inhibitor	0.06	0.12
Emetine dihydrochloride hydrate	Apoptosis	Apoptosis inducer; RNA-Protein translation inhibitor	0.38	0.05
Ivermectin	Cholinergic	Positive allosteric modulator of alpha7 neuronal nicotinic acetylcholine receptor; also modulates glutamate-GABA-activated chloride channels	0.25	0.94
PD173952	Kinase	PD173952 is a Src family kinase inhibitor.	0.64	0.1

Table-7: **List of LOPAC molecules with nanomolar potency against both *T. gondii* and *P. falciparum*.**

Name	Class	Known mechanism of action
10058-F4	Apoptosis	Inhibits c-Myc-Max interaction and prevents transactivation of c-Myc target gene expression.
G15	Bioactive Small Molecules	Selective GPR30 antagonist with little to no binding/activity towards classical estrogen receptors
R(+)-6-Bromo-APB hydrobromide	Dopamine	D1 Dopamine receptor agonist
Pyrocatechol	Cell Cycle	Carcinogen; causes DNA strand breakage
5-Nitro-2-(3-phenylpropylamino) benzoic acid	Cl- Channel	Potent Cl- channel blocker
NS 2028	Cyclic Nucleotides	Specific soluble guanylyl cyclase inhibitor

Table-8: List of LOPAC molecules showing delayed death by inhibiting plaque formation.

Growth inhibition studies with the MMV Box libraries: A total of 790 molecules from the Malaria Box and Pathogen Box MMV libraries were screened in the primary assay to identify potent growth inhibitors at 10µM. This resulted in the identification of 229 molecules with > 80% inhibition on *T. gondii* growth, from which 94 molecules were found to have nano molar potency (i.e., EC_{50} < 1µM). We also identified 304 molecules that had no effect on parasite growth (i.e., < 20% growth inhibition) (**Figure-2.8**).In case of *P. falciparum*, 563 out of 790 molecules were able to inhibit the parasite growth, and 266 of them have shown nano molar potency (i.e., EC_{50} < 1 µM).We then compared the EC_{50} for *P. falciparum* and *T. gondii* and identified 34 molecules with nano molar potency against both parasites. A list of these 34 molecules is given in the table in Appendix-II.

T. gondii plaque forming assays with MMV Box molecules*:* Molecules which had < 20% growth inhibition in primary screens were tested for their ability to inhibit plaque formation by *T. gondii* tachyzoites. Out of 303 molecules screened in this plaque forming assay, 67 molecules showed complete inhibition of plaque formation, indicating that they are causing delayed death of parasites (**Figure-2.9**). Few other molecules were found to reduce the plaque size with respect to controls, indicating that these were regarding growth rather than completely inhibiting it. We have quantified no of plaques and plaque size for each treatment and results have been plotted in **Figure-2.10.** A list of these molecules is given in the table in Appendix-III.

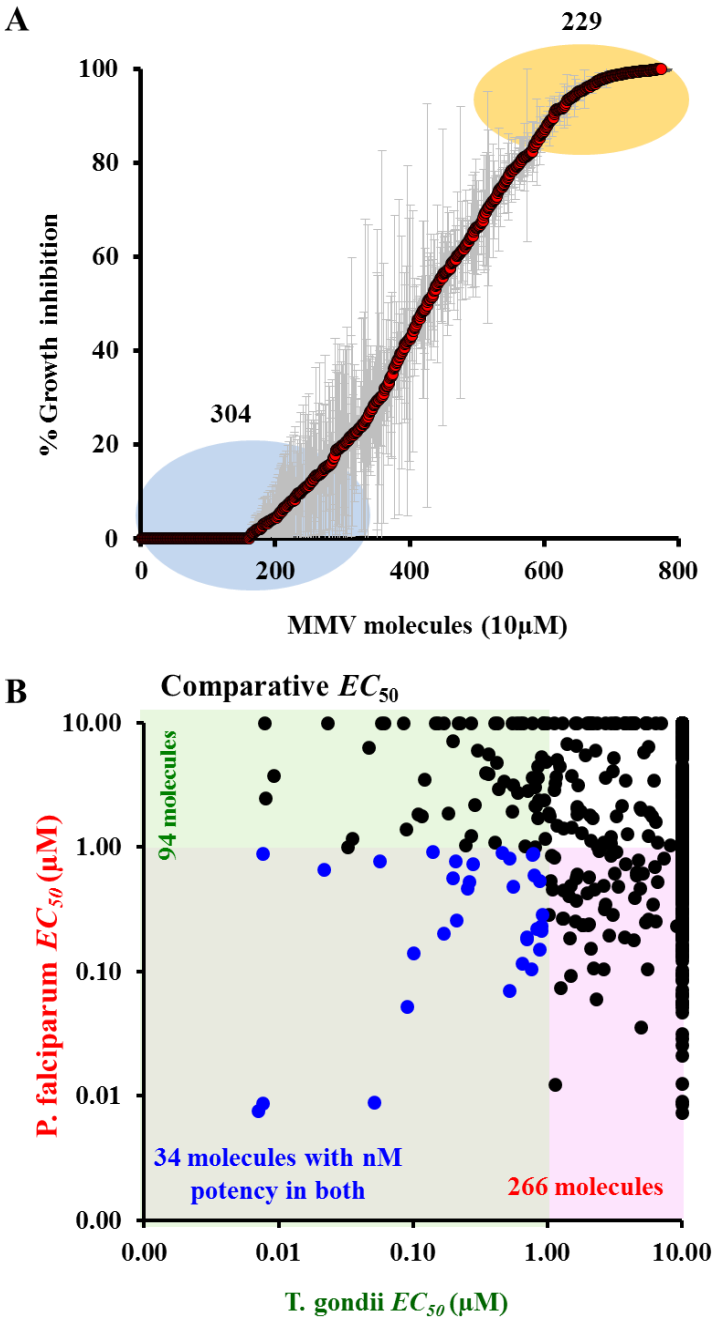

Figure-2.8. **Inhibitory effects of MMV molecules on *T. gondii* and *P. falciparum* growth**. Data shown is a combination of both Malaria Box and Pathogen Box molecules. **A)** Growth inhibition studies with *T. gondii* using 10µM fixed concentration of MMV molecules. The percentage growth inhibition, after normalizing with controls, is plotted for the various molecules. Atovaquone (1µM) treated parasites were used as positive control reference for 100% growth inhibition. **B)** Correlation of the EC_{50} of the MMV molecules against *P. falciparum* and *T. gondii*. The inhibitory potencies against asexual blood stage *P. falciparum* and tachyzoite stage *T. gondii* were compared. The 8 molecules with nano molar potency against both parasites are highlighted in blue.

Molecules whose EC_{50} was not determined (for those with <80% growth inhibition at 10 µM) are plotted at 10µM EC_{50} for the respective parasites.

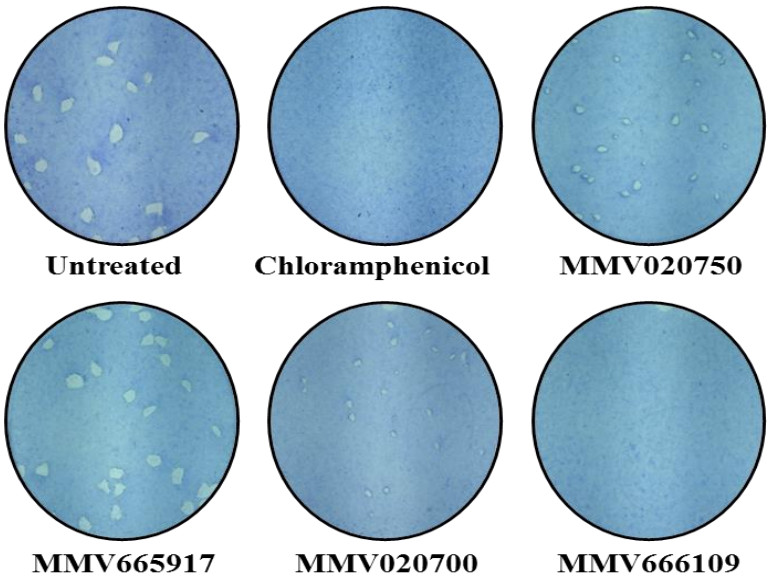

Figure-2.9: Representative images for plaque formation: Plaque formation in the presence of the inhibitor that either reduced plaque size or completely inhibited parasite growth. Chloramphenicol is used as a positive control for inhibition of plaque formation and untreated controls had 1% DMSO.

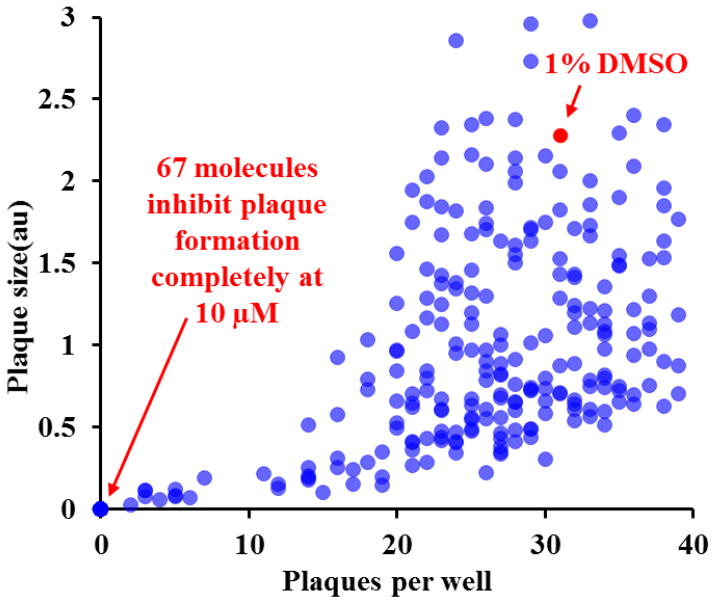

Figure-2.10: **Plaque-forming assays identify compounds that cause delayed death of *T. gondii***. From plaque assays, the number of plaques formed and the sizes of the plaques formed were determined. Average values for 1% DMSO-treated control samples are plotted in red. 67 were found to totally abolish plaque formation.

Discussion and conclusion

Parasitic diseases cause high mortality and morbidity in developing countries and pose an increasing threat to human health. Lack of vaccine against these parasitic diseases has made chemotherapy the only option for treatment. However, the anti-parasitic drugs currently in use have several limitations, including decreased efficacy due to drug resistance and potential toxicity, which has further worsened the scenario. With this regard, there is an urgent need for alternative anti-parasitic drugs. Developments in this area have been largely inadequate because of the major practical problems of conventional as well as alternative anti-parasitic drug screening assays. There is a strong need to evolve an appropriate screening technology, combined with combinatorial chemistry and computational biology, for increasing the efficiency of target-based drug discovery against protozoan parasites. In this regard we have developed a pipeline for chemical-genetic phenotyping, to identify novel drug targets in apicomplexan parasites.

The MMV Malaria Box molecules (Spangenberg *et al., 2013*) have been extensively studied for their inhibitory potential against asexual and sexual stages of *P. falciparum* (Van Voorhis *et al., 2016*). This resourceful antimalarial collection has also been screened against other parasitic species, such as kinetoplastids (Sykes and Avery, 2015, Kaiser *et al*., 2015), helminths (Ingram-Sieber *et al.*, 2014), *Babesia* (Paul *et al*., 2016), *Theileria* (Njuguna *et al*., 2014), *Cryptosporidium* (Bessoff *et al*., 2014), *Toxoplasma* (Boyom *et al.*, 2014), *Giardia* (Hart *et al.*, 2017), and *Entamoeba* (Boyom *et al.*, 2014).

A more recent molecule collection launched by MMV, known as Pathogen Box, is quite unexplored yet, but includes inhibitory molecules against a regime of pathogens. We have chosen Pathogen Box as our second chemical Library of interest as it's an interesting collection containing diverse chemical molecules active against various pathogens. Various

reports have already demonstrated the potential of Pathogen Box, by screening these molecules against the helminthic parasite *Haemonchus contortus* (Preston *et al*., 2016, Jiao *et al*., 2017), the fungal pathogen *Candida* spp. (Vila and Lopez-Ribot, 2016), many protozoan parasites such as *Neospora Canium* (Müller *et al*., 2017), *T. gondii* (Spalenka *et al*., 2018), *P. falciparum* (Rout *et al*., 2017, Tong *et al*., 2018, Calit *et al*., 2018), *Cryptosporidium parum* (Hennessey *et al*., 2018, Dhal *et al*., 2018), *Giardia lamblia* (Hart *et al*., 2017, Hennessey *et al*., 2018), *Entamoeba histolytica* (Mi-Ichi *et al*., 2018), the model nematode *Caenorhabditis elegans* (Partridge *et al*., 2018), and the bacterial pathogen *Burkholderia pseudomallei* (Ross *et al*., 2018).

Sigma LOPAC is a commercially available chemical library and is completely different in chemical class and diversity from the MMV libraries. One key advantage of using Sigma LOPAC bioactive compound collections is that the compounds are already known drugs or drug-like molecules and likely can be more readily modeled to increase potency while retaining good oral bioavailability. Medicinal chemistry approaches could be ideally carried out to modify existing molecules if promising parasite targets can be identified. This library was previously screened against multidrug resistant *P. falciparum* (Lucumi *et al*., 2010, Preuss *et al*., 2012), gametocytic stages of *P. falciparum* (Tanaka *et al*., 2013), and *T. gondii* (Murata *et al*., 2017).

The published experiments using all three libraries primarily focused on determining killing efficacies of the inhibitors in whole-cell assays but little or no information is available on their MOA in these parasites and only a very small subset of molecules in this library have been successfully mapped to their targets. Chemically induced phenotypes can facilitate downstream mechanistic studies, as they often serve as reliable indicators of cellular pathways perturbed by the molecule of interest. This requires customized screening

campaigns focused on the phenotype(s) of interest and is only possible when morphologically distinct cellular phenotypes can be linked to specific cellular pathways and targets. *P. falciparum* and *T. gondii*, two well-studied parasites, offer the opportunity to carry out such phenotypic screens, especially since experimental tools and reagents are readily available to dissect chemically induced cellular phenotypes. For instance, phenotypic features associated with impaired growth kinetics (i.e., fast versus delayed killing), host cytolysis (the endpoint of an intracellular replicative cycle of the parasite), and host invasion (to establish a new infectious cycle) are well characterized in both of these parasites. Furthermore, because of their shared evolutionary history, orthologous proteins in *P. falciparum* and *T. gondii* are often associated with similar cellular processes and, importantly, are likely to share sensitivity to inhibitors that affect these unique life stage events. This reasoning motivated us to undertake targeted phenotypic screening of these molecules to identify inhibitors in *T. gondii* and *P. falciparum*. Establishing the linkage between unique chemical scaffolds and their resultant cellular phenotypes on two evolutionarily related yet distinct parasites will provide an avenue for conducting detailed mechanistic studies on the organism of choice by biochemical, cell biological, and/or genetic approaches, as appropriate.

In this thesis, we have determined the parasite growth inhibition by molecules from the three libraries as primary phenotype for *P. falciparum* and *T. gondii*. This exercise also helped to confirm that the activities of the molecules obtained are in agreement with previously published data (Van Voorhis *et al., 2016,* Boyom et al., 2014, Spalenka *et al*., 2018). For *P. falciparum,* our results indicated overall agreement with the data previously published. However, in the case of *T. gondii,* we obtained results markedly different from previously published works (Boyom et al., 2014, Spalenka *et al*., 2018). The reported anti-toxoplasma studies on MMV Malaria Box molecules used a short-term (24-h) killing assay performed with *T. gondii* tachyzoites and identified only seven molecules with significant

anti-toxoplasma activity, of which only one, MMV007791, was reported to have nanomolar activity. In our assays, parasites were incubated for 48h with these molecules before they were checked for growth inhibition. We used a transgenic strain of *T. gondii* expressing the luciferase gene as a reporter, where the luminescence readout is more sensitive and reproducible than the previously used method of monitoring parasite killing.

Overall, we found that 342 of all molecules exhibited > 80% growth inhibition and 1152 had a < 20% growth inhibition effect on *T. gondii* tachyzoites at 10µM concentration. Of the 94 molecules that exhibited nano molar EC_{50} against *T. gondii*, 41 had nano molar potency against *P. falciparum* as well. Not much is known from the available literature regarding the mechanism of action of these 41 molecules.

Of these, 24 Malaria Box molecules, a few, MMV665941, MMV006389, and MMV665977, were reported to inhibit *P. falciparum* gametocyte development (Ruecker *et al*., 2014) and MMV665941 was found to have transmission blocking activity (Vos *et al*., 2015). In a separate study, MMV665886 was found to have translation inhibition activity against *Plasmodium* (Ahyong *et al*., 2016). Metabolomic profiling has revealed that MMV666596, MMV665977, MMV006309, and MMV665940 induce metabolic changes consistent with inhibition of pyrimidine biosynthesis, while MMV396669 and MMV000963 induce metabolic changes consistent with inhibition of hemoglobin metabolism (Allman *et al*., 2016, Creek *et al*., 2016). Further mechanistic studies of these potent molecules, especially in T. *gondii*, are yet to be performed.

From Pathogen box, 10 molecules were found to be active against both apicomplexan parasites, and these were previously shown to be active against other pathogens as well. MMV010576 is a 2-amino pyridine antimalarial molecule shown to be active against *Leishmania donovani* as well (Duffy *et al*., 2017). An analog of this molecule is included in

the Malaria Box. Surprisingly MMV010576 is also reported to be active against *C. parvum* and *G. lamblia* in cell proliferation assays (Hennessey *et al*., 2018). MMV019189 was reported to be active against *P. falciparum* in previous reports (Duffy *et al*., 2017). MMV021013, a 2-pyridyl-4-aminopyrimidine, was shown to be active against various parasites but demonstrates greater activity against *T. cruzi* (1.67μM) and *L. donovani* (1.48μM) than against *T. brucei* (3.51μM). Activity against *Plasmodium* and *Leishmania* has also been reported before for this molecule (Chen *et al*., 2006, Musonda *et al*., 2009); however, activity against *T. cruzi* has not been recorded within the scientific literature. Based on chemical structure, the cellular target of these compounds in *Plasmodium* is proposed to be methionine aminopeptidase. Therefore, these compounds could also target peptidases in *T. cruzi* and *T. gondii* although this needs to be confirmed (Duffy *et al*., 2017).

MMV023227 is reported to be a potent transmission blocker in *P. falciparum* (Calit *et al*., 2018). MMV024397 was reported to be an antimalarial molecule (pathogen box data) and MMV671636 was found to be a filariasis and onchoceriasis inhibitory compound, and is also reported to inhibit *N. canium* intracellular growth and replication. It's a quinolone compound and its target is predicted to be the mitochondrial bc1 complex (Müller *et al*., 2017 and *mmv.org*). MMV675968 is known to target the *Cryptosporidium* dihydrofolate reductase (DHFR) (Popov *et al*., 2006) and is structurally similar to anti-malarials that are known to target DHFR (Gebauer *et al*., 2003). It is therefore likely that MMV675968 targets *Plasmodium* DHFR (Duffy *et al*., 2017). This compound was also shown to be active against mycetoma (Lim *et al*., 2018). MMV676477, one of the potent inhibitor from our screen, has been shown to be active against *T. gondii* and *N. canium* in other reports (Spalenka *et al*., 2018, Müller *et al*., 2017). MMV688754, an oxime analogue of trifloxystrobin, inhibits kinetoplastid parasites and was also shown to be active against *T. gondii* and *P. falciparum*. MMV689480 is a reference compound, and is an anti-protozoan

molecule known as buparvaquone, which is mechanistically similar to the oxidative phosphorylation inhibitor atovaquone. It is the only drug available currently for clinical treatment of theileriosis in cattle, which is another apicomplexan parasite of economic importance.

Hits identified to be active against both parasites form LOPAC library includes artemether, which is a methyl ether derivative of artemisinin, and is found to be active against both the parasites, which is in accordance with the already published data (Meshnick, 1998, Berens *et al*., 1998). It is more potent towards *P. falciparum* (EC_{50} 20nM) when compared to *T. gondii* (EC_{50} 300nM). This difference in potency may be due to permeability issues related to host cells. The emetine dihydrochloride hydrate molecule is used in the laboratory to block protein synthesis in eukaryotic cells by binding to the 40S subunit of the ribosome and inhibiting its activity. Emetine induces hypotension in mammals by blocking adreno receptors. Also, emetine was identified as a specific inhibitor of HIF-2α protein stability and transcriptional activity. It's been shown to inhibit P. *falciparum* growth *in vitro* (Lucumi *et al*., 2010**)** and our assays also have picked it up as a potent inhibitor of both the parasites. Ivermectin is a derivative of the avermectins, a family of macrocyclic lactones produced by the filamentous bacterium *Streptomyces avermitilis*. With ivermectin, we found a significant inhibitory effect against both the parasites. In our cytotoxicity experiments, we found that ivermectin also has cytotoxic effects against HFF cell at higher concentrations. Other two compounds with antiparasitic activity from the LOPAC collection are cell-signaling inhibitors in mammalian cells; one is a K^+ channel inhibitors and another is nitric oxide synthase inhibitor. These compounds are less explored and its target in parasites needs be determined. Further mechanistic studies of these potent molecules, especially in *T. gondii,* have yet to be performed.

Next, among the molecules that showed < 20% growth inhibition in *T. gondii* killing assays, we have identified 67 that completely inhibited plaque formation, suggesting that they are causing delayed death of the parasite. Taken together, our results provide comprehensive documentation of selective effects of MMV Box and Sigma LOPAC molecules against *P. falciparum* and *T. gondii*. By employing complementary screening techniques, we have prioritized a few of these inhibitors for further studies on their MOA *via* parasite specific phenotypic effects. A majority of the hits identified are "drug-like" molecules, which in addition to having antimalarial efficacy, also have chemical characteristics that make them amenable to subsequent medicinal chemistry work. Further investigations to dissect their mode of action by combining biochemical and genetic approaches will facilitate their exploitation for therapeutic use against malaria and toxoplasmosis.

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Chapter 3

Chemo-phenotypic screens to identify the unique cellular level phenotypes exhibited by inhibitor treated cells

Introduction

As detailed in Chapter-2, the chemical screening efforts have helped us to identify molecules on the basis of their inhibitory activity on parasite growth. We have tested these molecules in two closely related parasitic species, *P. falciparum* and *T. gondii*, to identify and exploit molecules that generate complementary phenotypes. Due to their shared evolutionary history, a large percentage of genes encoded by these two parasites are orthologous; 2482 *P. falciparum* genes (~50% of total) and 2591 *T. gondii* genes (~30% of total) (Li *et al*., 2003, Chen *et al*.,2006). Many of these conserved genes make up core components of indispensable cellular processes required for development, replication, egress or invasion in both parasites. Hence, it is reasonable to expect comparable phenotypic responses when exposed to a molecule that target a conserved ortholog. For example, macrolide antibiotics affecting plastid housekeeping functions were shown to have similar phenotypic effect in *P. falciparum* and *T. gondii* (McFadden *et al.*, 1996, Pfefferkorn *et al*., 1992, Fichera *et al*., 1995, Beckers *et al*; 1995, Rogers *et al*., 1992).

Furthermore, the apical organelles, such as the rhoptries, micronemes, and many of the resident proteins in these compartments involved in invasion are orthologous and conserved between these parasites (Blackman and Bannister, 2001, Singh and Chitnis, 2012,). Therefore, inhibition of these proteins can be expected to have similar cellular phenotypic effects on impaired host invasion in both parasites. Work from earlier reports has shown that perforins and proteases (Roiko and Carruthers, 2009, Hale *et al*., 2017), which help in active egress of the parasite from infected host cells, are conserved in both *Plasmodium* and *Toxoplasma.* In addition, despite the differences in the mammalian host cell types these parasites invade, they appear to co-opt the same host factors to induce host cytolysis and egress (Chandramohanadas *et al*., 2009), highlighting deeply conserved similarities at the molecular level. This chapter describes the results from two phenotypic assays in *T. gondii*-

inhibition of apicoplast segregation into daughter cells resulting in delayed death of the parasite and inhibition of parasite egress from host cells when stimulated with a calcium ionophore. A detailed overview of the experimental strategy used for molecule selection and screening for the desired phenotype is shown in **Figure-3.1**

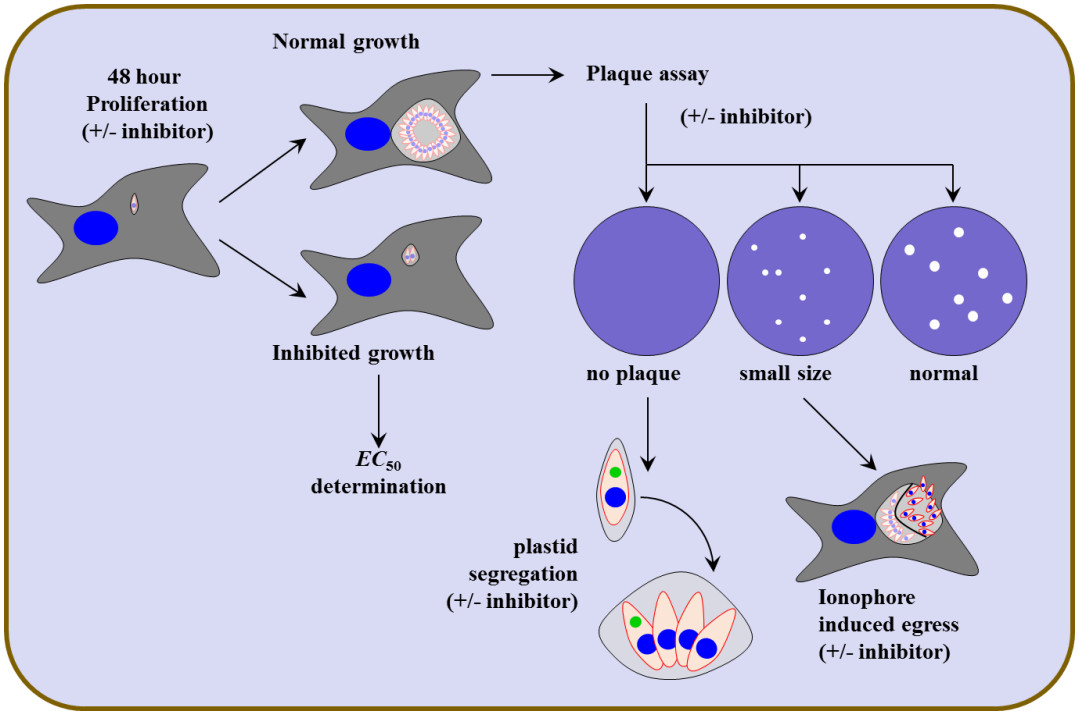

Figure-3.1: Overview of the experimental strategy for the inhibitor identification screens. The left side of the panel depicts the primary screens (described in Chapter-2) and the right side of the panel depicts the strategy used to select molecules for the phenotypic screens. The work flow shown here was common to the three chemical libraries screened in this study.

Material and Methods

Molecular protocols: In order to carryout microscopic studies on apicoplast dynamics during parasite cell division, tachyzoite stage parasites constitutively expressing a YFP tagged version of the plastid localized triose phosphate isomerase II gene (RH-*Tg*tpi-II-YFP) was used. Since the TPI-II protein is naturally targeted to the apicoplast, in this transgenic parasite, the organelle is marked by YFP fluorescence. The genomic locus tagging plasmid

construct used to generate transgenic parasites expressing *Tg*tpi-II-YFP was made as follows. Genomic DNA was isolated from tachyzoite stage *T. gondii* with a commercial kit (Qiagen) in accordance with the manufacturer's protocol. The Topo 2.1 cloning vector (Invitrogen) was modified to make a 3' YFP tagging plasmid. PCR-amplified 1.7-kb *T. gondii* genomic DNA corresponding to the 3' region of the *Tg*TPI-II gene locus (TGME49_233500 and TGME49_chrVIII, 2,682,525 and 2,688,704), which includes the codon for the last amino acid but excludes the stop codon, was cloned as a HindIII and AvrII fragment. The YFP coding sequence, along with the *Tg*DHFR 3' UTR region, was then cloned downstream of the *Tg*TPI-II genomic region. The DHFR-TS selection cassette, used to obtain a stable line by pyrimethamine selection, was cloned into the tagging plasmid as a NotI fragment. Prior to parasite transfection, the tagging plasmid was linearized with the BstXI enzyme, which cuts in the middle of the 1.7-kb *Tg*TP-II genomic fragment (**Figure-3.2**).

Generation of transgenic parasite line: Freshly isolated tachyzoite stage *T. gondii* were washed and resuspended in complete parasite culture media. 50 µg linearized plasmid DNA dissolved in 100µl of media was mixed with 300µl of parasite suspension (~$1x10^7$ cells), and the mixture was electroporated using a Gene Pulser electroporation system (Biorad) with capacitance and voltage settings at 10 Faraday and 1.5 kV respectively. Transfected parasites were then allowed to infect a fresh layer of HFF cells and incubated for 12 hours in optimal growth conditions. Infected HFF monolayers were then washed once, and 1µM pyrimethamine was added to culture medium and incubated for 48 hours to obtain stable transgenic lines, which were then cloned out by limiting dilution method.

Plaque formation and delayed death assays in T. gondii: Plaque forming ability of *T. gondii* tachyzoites (RH strain) was tested to detect 'delayed death' effect for Malaria Box molecules having < 20% growth inhibition on tachyzoite stage *T. gondii* in the primary inhibitor identification assays (see Chapter-2). Confluent human foreskin fibroblast (HFF) monolayer

in six-well plates was inoculated with parasites for plaque formation. Molecules were plated at 10µM, and 1% DMSO-treated cells were used as controls. The infected cultures were left undisturbed for 10 days, after which they were processed for visualization of plaque formation. The total number of plaques formed per well and the average plaque area were quantified for each treatment. Molecules, which inhibited plaque formation completely, were selected for image-based analysis to identify those affecting apicoplast segregation during parasite cell division. Molecules, which reduced the plaque size significantly after 10 days, with respect to untreated parasites, were selected for testing their parasite egress blocking activity.

Microscopy based apicoplast mis-segregation phenotype assay in T. gondii: The transgenic RH-*Tg*tpi-II-YFP parasites, were used to track the apicoplast phenotype by microscopy. First, tachyzoite stage parasites ($5x10^3$) were allowed to invade a confluent monolayer of HFF cells (1^{st} vacuole) in 96 well plates, in the presence of selected molecules (10µM) identified to have delayed death effect on the parasite. After 48 hours of incubation in optimal growth conditions, parasites were harvested by trypsin treatment followed by syringe passing of the infected cells through a 25-gauge needle to release free extracellular parasites. These parasites were then added to a fresh monolayer of HFF cells grown on coverslips in 24 well plates, to initiate a second round of invasion (2^{nd} vacuole), again in the presence of 10µM inhibitor. After ~20 hours of growth in optimal conditions, the coverslips were fixed with 3.5% paraformaldehyde and stained with DAPI to visualize cell nuclei, and mounted on a glass slide using fluoromount (Sigma). The coverslips were then imaged using a 63X objective fitted to an inverted fluorescence microscope (Carl Zeiss). Apicoplast associated YFP and nuclear DAPI were imaged using the excitation/emission filter combinations 514nm/527nm and 350nm/470nm, respectively.

Calcium ionophore mediated T. gondii egress phenotype assays: The calcium ionophore A23187 was used to induce egress of intracellular tachyzoites as previously reported (Eidell *et al.*, 2010). Briefly, 24 hours post invasion (hpi) of host cells, when the vacuoles contain between 8 to 16 parasites, the culture plates were removed from the incubator and allowed to equilibrate to room temperature for 5 minutes before identifying appropriate live parasite vacuoles by microscopy using a 40X objective fitted to an inverted bright field microscope (Primo Vert, Zeiss). 2µM of the ionophore was added to the culture medium and the selected vacuoles were imaged by time-lapse microscopy up to 10 minutes. The time taken by the tachyzoites to egress out of the host cells following the addition of the ionophore was monitored and recorded.

Results

Generation of transgenic parasite line expressing Tgtpi-II-YFP: *Tg*tpi-II-YFP expressing transgenic parasite lines was generated as described in the method section. The proper tagging of the *Tg*tpi gene at the 3' end with the cDNA coding for the YFP marker was confirmed by genomic DNA PCRs. Microscopic images were used to confirm the localisation of *Tg*tpi-II-YFP in the apicoplast of these parasites (**Figure-3.2).**

Studying the apicoplast mis-segregation effect of molecules causing delayed death in T. gondii: In the primary hit identification assays, 67 molecules from the two MMV Box libraries and 848 molecules from the Sigma LOPAC collection showed virtually no effect on parasite growth (i.e., < 20% inhibition at 10µM in the 48 h killing assays; Chapter-2). We were interested in evaluating if any of these molecules induce delayed death in *T. gondii* tachyzoites.

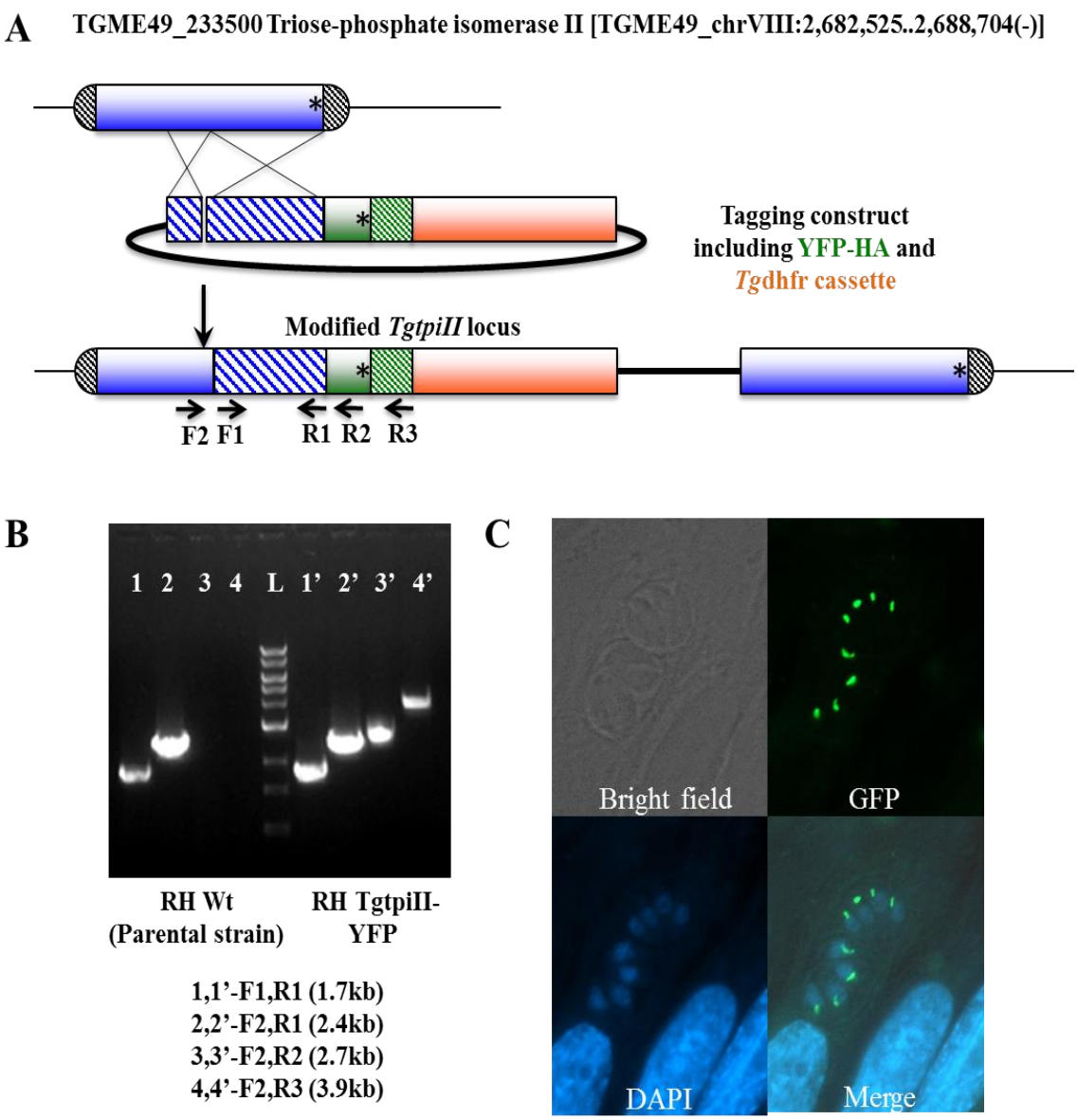

Figure-3.2: Generating the RH-*Tgtpi-II-YFP* transgenic parasite line. A) Schematic diagram depicting the homologous recombination mediated genomic locus tagging of the *Tg*tpi-*II* gene with YFP. The tagging construct also contains the dhfr selection cassette, which allows selection of transgenic parasite lines with pyrimethamine. The primer sequence used for amplifying the genomic fragments and verifying the insertion is included in the primer table (Appendix-IV). **B)** Genomic PCR based confirmation of proper tagging of YFP at the locus of interest. Lanes 1, 2, 1' and 2' show PCR fragments that are expected from parental as well as transgenic RH strain *T. gondii*. Lanes 3, 4, 3', and 4' show the presence of tag specific PCR products only in the RH-*Tg*tpi-II-YFP transgenic parasite line. Lane L, 1 Kb marker DNA ladder. **C)** Microscopic examination of YFP expression and its localisation in the apicoplast organelle.

Delayed death of apicomplexan parasites is a well-studied phenotype for antibiotics such as chloramphenicol and clindamycin which targets the ribosomal machinery in the apicoplast (Fleige and Soldati-Favre, 2008, Dahl and Rosenthal, 2008, Foth and McFadden, 2003). In this case, parasites continue to replicate within the first parasitophorous vacuole (PV) following inhibitor treatment and exhibit no apparent growth inhibition until they egress and reinvade a naive host cell, where they fail to replicate and die in the second vacuole. The molecular mechanism behind this delayed death phenomenon appears to be associated with inhibition of housekeeping functions in the apicoplast (Beckers *et al*., 1995, He *et al*., 2001, Fichera and Roos., 1997, Köhler *et al*., 1997).

To evaluate possible delayed-death effects of the molecules that showed no significant effects on *T. gondii* in 48-h killing assays, plaque-forming assays were carried out. Tachyzoite stage parasites were allowed to form plaques (zones of host cell lysis formed by multiple rounds of invasion and egress by parasites) on confluent host cell monolayers in the presence of inhibitors. A spectrum of growth phenotypes with respect to plaque number and size was obtained in the plaque assays. Most of the molecules tested did not significantly affect the number of plaques formed with respect to DMSO-treated control cultures and averaged between 20 and 35 plaques per well (for MMV molecules). In the case of a few molecules (MMV666597, MMV665882, MMV146306, MMV688330, MMV676584, MMV676603, MMV676597, MMV687188, MMV687699, MMV023969), we observed less than 10 plaques each and these were dramatically reduced in size (~0.1 U compared to a control plaque size of ~1.58 U). For many other inhibitors, even though the plaque counts were similar to those of control cultures, the plaque size was reduced to at least half of the control size. This can be due to either altered intracellular growth or replication of the parasite in the presence of the inhibitor or a delay in parasite egress from infected host cells, resulting in delayed progress in plaque formation. We identified 67 molecules, which

inhibited plaque formation from both of the MMV Box chemical libraries, which were further tested using apicoplast mis-segregation assays.

In case of the Sigma LOPAC library only presence or complete absence of plaque was scored (disregarding plaque size effects due to the sheer number of molecules that needed to be screened). Based on this criterion, we were able to identify 6 molecules, which clearly and consistently inhibited plaque formation by *T. gondii* tachyzoites. These molecules were included in the apicoplast mis-segregation phenotype assays to evaluate if their MOA was mediated *via* inhibitory effects on the plastid.

Identification of molecules inhibiting egress of intracellular T. gondii tachyzoites: We selected 45 molecules that caused a reduction in plaque size to test their inhibitory effects on *T. gondii* egress. For this, we adopted the widely used assay in which the calcium ionophore A23187 is used to induce intracellular tachyzoite egress (Endo *et al*., 1982, Eidell *et al.,* 2010) and monitored the delay in time before the rupture of individual vacuoles and parasite release. Parasites were allowed to infect and replicate in HFF monolayers in the presence of either 10µM inhibitor or 1% DMSO (as a negative control) for 24 h, followed by ionophore addition to induce egress. The time required for parasite egress from individual vacuoles was recorded and compared between DMSO- and inhibitor-treated parasites. The average egress time obtained for 1% DMSO-treated control parasites was ~1.8 min. In the case of inhibitor-treated parasites, we looked for an average egress time of at least 4 min to identify potential egress inhibitors. Of the 45 molecules tested, 40 had no significant inhibitory effect on ionophore-induced parasite egress, suggesting that the reduced plaque size observed in the presence of these molecules is primarily due to diminished parasite fitness rather than a delay in parasite egress.

However, one molecule, MMV001239, significantly inhibited parasite egress (**Figure-3.3**). It is interesting that MMV001239 exhibited time-dependent egress-blocking activity, as the delay in egress was much less pronounced upon treatment with 10µM compound for 6 h (average egress time, ~3.3 min) than upon treatment for 24 h, where parasites failed to egress from most of the vacuoles even after 10 min following ionophore treatment (**Figure-3.3**).The egress inhibitory effect of MMV001239 on *T. gondii* appears to be very specific, as this molecule has no effect on parasite growth, and plaque assays revealed the formation of plaques only slightly smaller than those of the untreated control (**Figure-3.3**). Reduced plaque size may result from delayed egress of parasites following each round of infection during the course of plaque formation. Thus, it appears that MMV001239 is a genuine inhibitor of *T. gondii* egress from host cells.

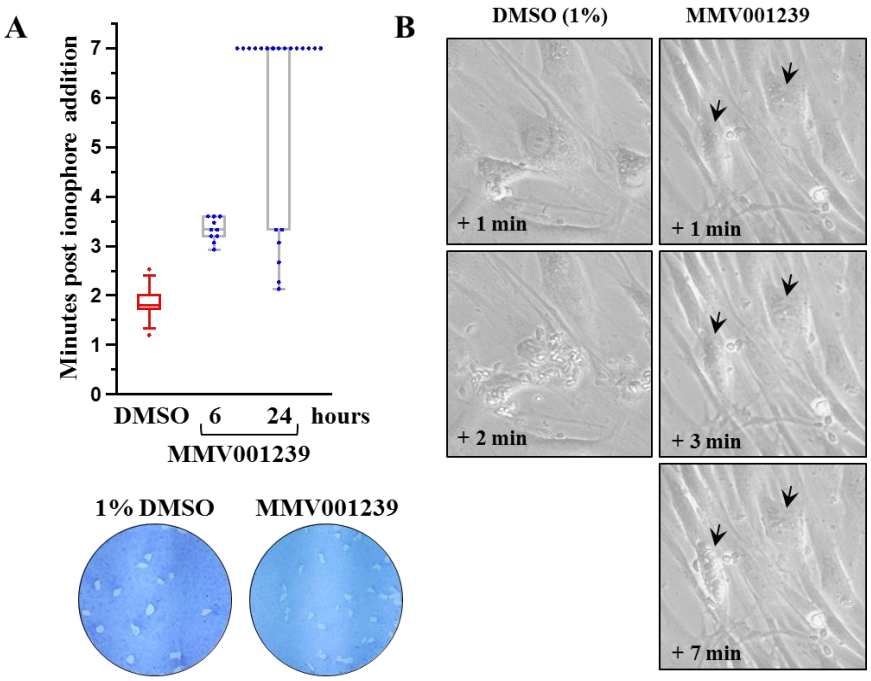

Figure-3.3: Inhibition of ionophore-induced egress by MMV001239: A) Inhibition of ionophore-induced egress by MMV001239 with a median egress time of >3 min and >10 min for 6 and 24 h of treatment with the compound at 10 µM, respectively. The median egress time of 1% DMSO-treated control parasites is 1.8 min. At the bottom are the plaques with reduced size formed in the presence of MMV001239, in comparison to 1%

DMSO-treated controls. (B) Time-lapse microscopic images showing a delay in ionophore-induced parasite egress following 24 h of treatment with MMV001239 (arrows indicate the intact parasitophorous vacuoles.)

Interestingly, in a recent study, it was shown that MMV001239 targets the lanosterol-14-demethylase enzyme in *T. cruzi* (Ottilie *et al.,* 2017). Even though *T. gondii* does not encode an ortholog of this enzyme, it is likely that MMV001239 acts by interfering with membrane lipid dynamics, which is known to facilitate parasite egress from host cells. Although MMV001239 may not be useful as an anti-toxoplasma agent because of its inability to abrogate parasite growth, we expect that further mechanistic studies with this molecule can help in dissecting the parasite egress pathway and identifying novel targets.

T. gondii egress inhibition studies with MMV molecules capable of inhibiting egress of P. falciparum merozoites: Host cell cytolysis pathways of *T. gondii* and *P. falciparum* are known to have conserved aspects (Blackman and Carruthers, 2013, Gubbels and Duraisingh, 2012, Dowse *et al.,* 2008, Friedrich *et al.,* 2012 and Sibley, 2010). Therefore, we wanted to test if the MMV molecules affecting the egress of *P. falciparum* merozoites from infected erythrocytes are also capable of inhibiting the egress of *T. gondii* tachyzoites. In a parallel study from our collaborators laboratory, 43 molecules were found to potently block merozoite egress in *P. falciparum* schizont maturation assays. We therefore tested the egress blocking effect of these molecules in *T. gondii* in the ionophore-induced egress assay. At 10μM, 12 of the 43 molecules had a cytotoxic effect on HFF cells, which disqualified them from further *T. gondii* egress studies. Of the remaining molecules, 5 Malaria Box molecules (MMV396719, MMV019127, MMV006427, MMV007617 and MMV006429) and 5 Pathogen Box molecules (MMV020670, MMV023183, MMV020081, MMV026356 and MMV032967) were found to have potent inhibitory effect on *T. gondii* egress. The egress time for these molecules was > 7 minutes (post ionophore treatment) and > 80% of the *T.*

gondii vacuoles remained intact (**Figure-3.4**). Interestingly few of these molecules also reduced the plaque size in 10 days plaque assays as compared with the untreated (1% DMSO treated) control (**Figure-3.5**).

Notably, all of these potent egress inhibitors form MMV Box libraries, except MMV019127 and MMV026356, are known to disrupt sodium and pH homeostasis in *P. Falciparum* (Lehane *et al*., 2004, Spillman and Kirk, 2015, Dennis *et al*., 2018). The fact that none of these molecules were subjects of egress studies until now in the broader context of antiparasitic target discovery highlights the utility of comparative screening efforts such as those undertaken in this study.

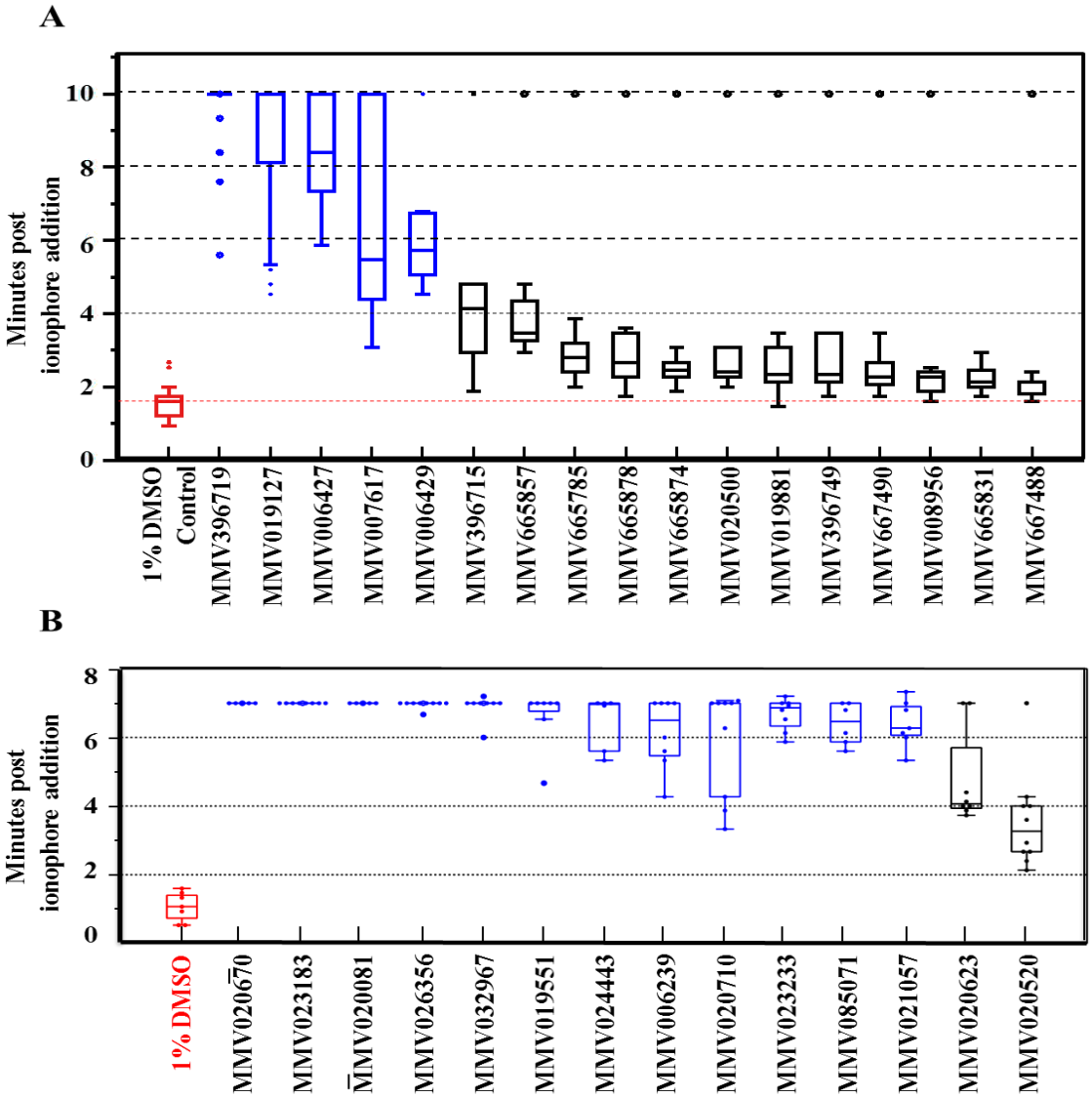

Figure-3.4: Inhibition of calcium ionophore-mediated egress of *T. gondii* tachyzoites. (A) Whisker plots of the timing of ionophore-induced egress of parasites following 24 h of treatment with selected Malaria Box molecules at 10µM. Red, 1% DMSO-treated control cells; blue, inhibitors for which the median egress time was > 4 min. B) Whisker plots of the timing of ionophore-induced egress of parasites following 24 h of treatment with selected Pathogen Box molecules at 10µM. Red, 1% DMSO-treated control cells; blue, inhibitors for which the median egress time was > 4 min

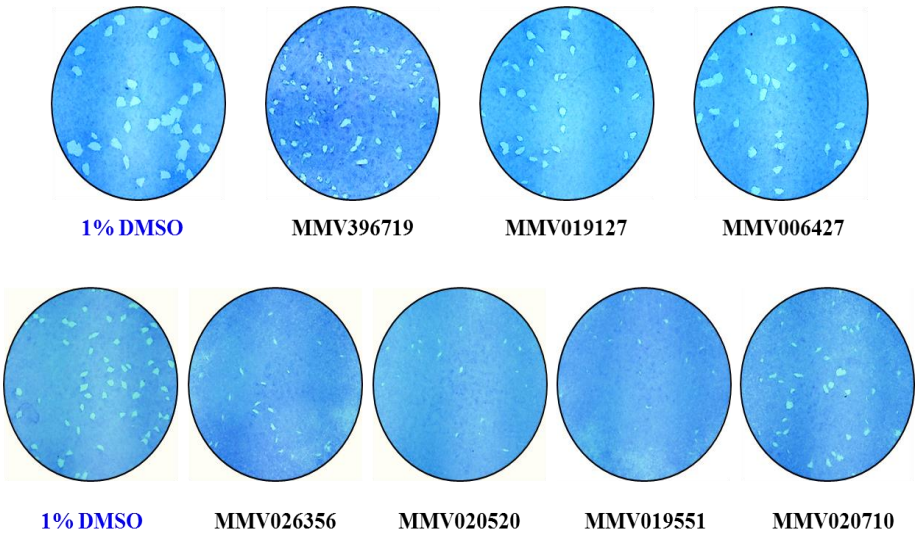

Figure-3.5: **Egress inhibitors reduce the size of plaques formed by tachyzoite stage *T. gondii***. Top panel depicts Malaria Box molecules and bottom panel depicts Pathogen Box molecules. Both experiments have different controls, as comparison of plaque size with untreated control was made within each experiment.

Effect of delayed death molecules on apicoplast segregation in T. gondii: Delayed death phenomenon in apicomplexan parasites is primarily attributed to inhibition of apicoplast housekeeping functions. A well-documented phenotypic consequence of this effect is characterized by the failure to properly segregate the apicoplast to the newly forming daughter cells during cell division. This results in a parasitophorous vacuole containing a mixture of daughter cells with and without the apicoplast organelle. This phenotype has also been validated by genetic intervention (He *et al.*, 2001). The plastid-less parasites survive until they egress out of the current host cell and invade a new host cell, where they die. This

phenomenon can be tracked phenotypically by microscopic observation to identify parasitophorous vacuoles, which contain both plastid-bearing and plastid-less parasites.

We have tested for possible plastid mis-segregation effects for the 73 molecules that were selected because of the delayed death effect they had on *T. gondii*. In this assay, we first allowed *T. gondii* tachyzoites to invade and replicate within HFF monolayers in the presence of 10µM inhibitor for 24 hours. Parasites were then physically egressed, and host cell free parasites were allowed to invade a naive confluent monolayer of HFF cells grown on glass cover slips, in the continued presence of 10µM inhibitor. 24 hours later, the glass coverslips were fixed with 4% paraformaldehyde and processed for microscopy. A parasite line, expressing the triose phosphate isomerase II gene tagged with YFP (*Tg*tpiII-yfp), was used in this experiment to visualize the apicoplast due to the fluorescence emitted by YFP targeted to the organelle. Among the 73 molecules tested in this assay, we observed plastid segregation defect in case of 4 Malaria Box molecules, 23 Pathogen Box molecules and 1 LOPAC molecules (**Figure-3.6**). Chemically, most of these molecules are distinct from the antibiotics that are known to cause plastid mis-segregation and delayed death in apicomplexan parasites and few of these are either reference molecules or analogues of other antibiotics. We have shortlisted these molecules for further mechanistic studies and to identify the target for these molecules.

Discussion and conclusion

Prior success with phenotypic screens have proven chemical genetics is a valuable tool to understand apicomplexan biology. Using small molecule inhibitor screens to identify inhibitors of parasite growth is still an emerging area, both in pharmaceutical industries as well as in academia. Efforts taken to develop robust screens addressing the complex biology of parasite and converting these into high throughput formats, are far and few.

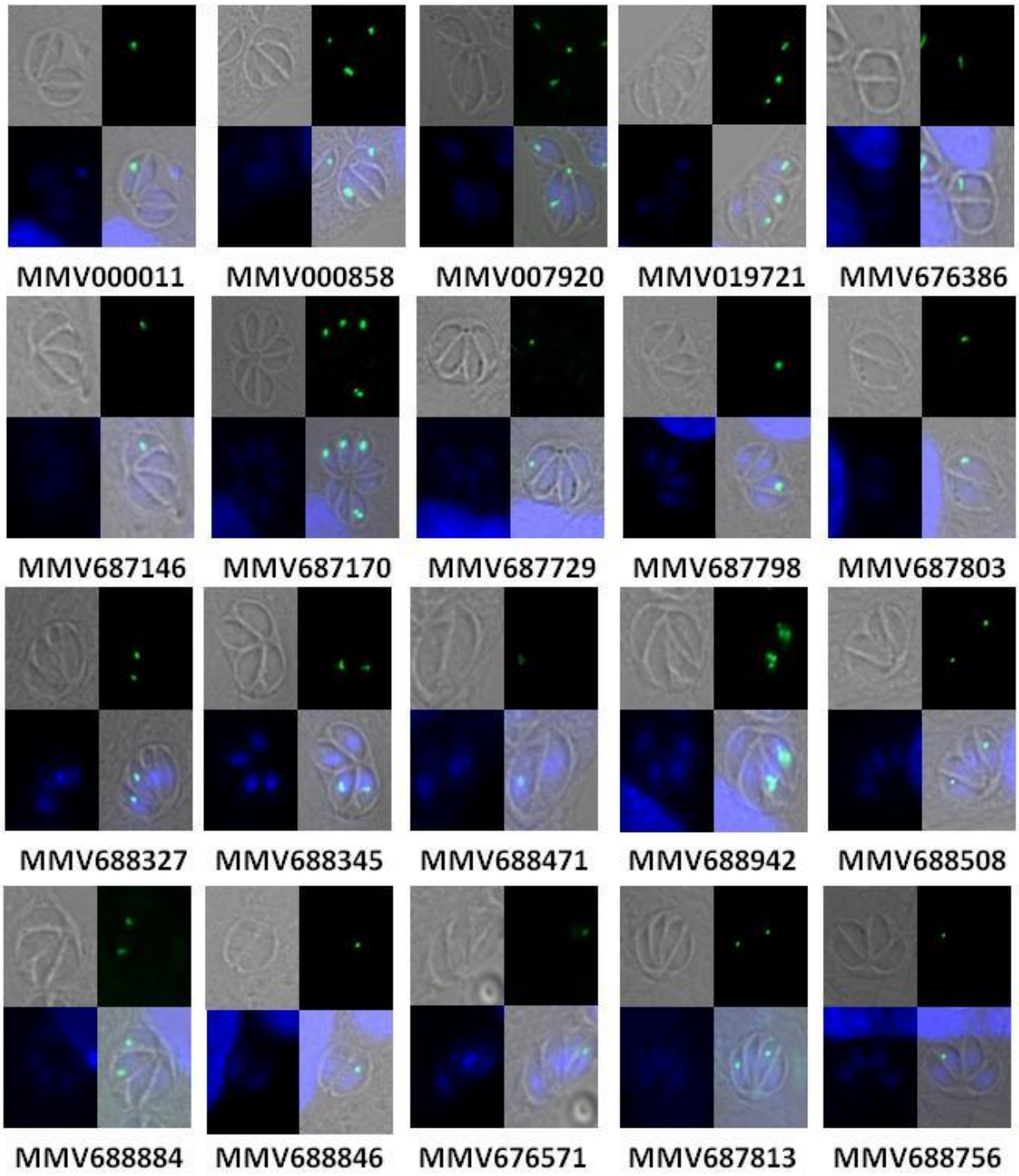

Figure-3.6: **Delayed death causing molecules in *T. gondii*:** Images from some of the representative molecules from MMV collection that caused delayed death and does plastid mis-segregation in intracellular parasites.

Newer methods to improve the cell based screens will be the key for better understanding of the unexplored parts of parasite biology as well to find new therapeutic options for the disease.

Towards this end, we have developed microscopic imaging based phenotypic screens, to monitor apicoplast mis-segregation and egress inhibition using *T. gondii* as a model parasite. Using these phenotypic assays we have explored the anti-toxoplasma activity of three different libraries –the MMV supported Malaria Box and Pathogen Box libraries and the Sigma LOPAC collection. Among the molecules that were found to have no significant effect on parasite growth (i.e., < 20% growth inhibition in killing assays), we identified 73 molecules that completely inhibited plaque formation by *T. gondii*, suggesting that they cause delayed death of the parasite. Notably, most of Malaria Box molecules have potent and immediate-killing anti-plasmodial activity, indicating that only a few of these molecules may be acting *via* the delayed death mechanism. However, the Pathogen Box molecules are more diverse in their chemistry and are also known to affect a variety of other pathogens, both eukaryotic and prokaryotic. Therefore we expected to find more molecules with delayed killing effect on *T. gondii*. The mechanism of delayed killing of apicomplexan parasites has been extensively studied and is linked to the inhibition of apicoplast housekeeping functions (Dahl and Rosenthal, 2008, Foth and McFadden, 2003). Examples of delayed-death inhibitors include antibiotics such as azithromycin, clindamycin, and doxycycline, which target the 70S ribosome, and ciprofloxacin, which targets the apicoplast-associated DNA gyrase (Fleige and Soldati-Favre, 2008). These antibiotics are equally effective in causing delayed death in *P. falciparum* and *T. gondii*, although phenotypic differences exist (Dahl and Rosenthal, 2007).

We identified four molecules from the MMV Malaria Box library (MMV008455, MMV020885, MMV019199, and MMV666109) and 23 molecules from MMV Pathogen Box library (MMV688942, MMV688756, MMV676571, MMV688471, MMV019721, MMV676386, MMV000011, MMV020321, MMV000858, MMV687803, MMV688327, MMV688846, MMV688508, MMV687248, MMV687146, MMV687170, MMV688844, MMV687798, MMV007920, MMV688345, MMV687729, MMV687813 and

MMV688990). Along with the MMV molecules, we also identified 1 molecules from the Sigma LOPAC library, which induced apicoplast mis-segregation during daughter cell formation in *T. gondii*.

MMV688942 is Bitertanol and is used as a fungicide. It was found to target Ergosterol biosynthesis in eumycota species (Berg *et al*., 1986). MMV676571 is a fenarimol analogue and its target is predicted to be steroid biosynthesis in Leishmania species (Zeiman *et al*., 2008). MMV688756 is commonly called as sutezolid, which is an oxazolidinones, and currently under clinical trial for the treatment of tuberculosis. MMV688327 and MMV688508 also belong to the same chemical class as oxazolidinones, are a new group of antibiotics. These drugs are active against a large spectrum of Gram-positive bacteria, including methicillin- and vancomycin-resistant staphylococci, vancomycin-resistant enterococci, penicillin-resistant pneumococci and anaerobes. Oxazolidinones inhibit protein synthesis by binding at the P site of the bacterial ribosome 50S subunit (Shinabarger *et al*., 1999, Jeong *et al*., 2018, Low *et al*., 2017). MMV000011 is a doxycycline derivative and hence their effect over apicoplast segregation is quite evident. MMV687798 is a reference compound levofloxacin, abroad-spectrum, fluoroquinolone antibiotic with antibacterial activity. Levofloxacin diffuses through the cell wall and acts by inhibiting DNA gyrase (prokaryotic topoisomerase II), an enzyme required for DNA replication, RNA transcription, and repair of bacterial DNA. Inhibition of DNA gyrase activity leads to blockage of prokaryotic cell growth, and hence it is a useful internal validation for our screening strategy, as this inhibitor, which is known to target the apicoplast, came out as a hit from our plastid phenotype screens.

MMV688345 is an analogue of already known antifolate agent and its target is presumed to be the dihydrofolate reductase dehydrogenase thymidilate synthase (DHFR-TS) enzyme involved in folate biosynthesis and 1 C cycle (Rosowsky *et al*., 1993). It is surprising to find

a DHFR inhibitor as delayed death causing molecule as antifolates are potent fast killing molecules in general. Therefore we expected it will be interesting to study the MOA for this molecule. MMV007920 is a benzoxazole-containing compound previously identified in a screen for agents that inhibit late stage gametocyte development in *P. Falciparum* (Duffy *et al*., 2017). MMV688471 was shown to inhibit IMPDH in *C. parvum* and have shown no inhibitory activity against *T. gondii* growth (Gorla *et al*., 2013). MMV019721 and MMV020321 were shown to be inhibitors of lactate dehydrogenase of *P. falciparum* (ChEMBL data; www.ebi.ac.uk/chembl/beta/). MMV676386 and MMV687803 were identified as hits in a *P.falciparum* delayed death screen (Data in PubChem; David Fidock and Eric Ekland, Columbia University).

MMV000858 is ion homeostasis inhibitor and was shown to inhibit the growth of enteric pathogens *Giardia* and *Cryptosporidium.* (Dennis *et al*., 2018, Hennessey *et al*., 2018) MMV688846 and MMV687146 are inhibitors of *Mycobacterium* and their target is predicted to be Trehalose monomycolate exporter MmpL3 protein (Low *et al*., 2017). MMV687248 is another anti mycobacterial compound, a 3, 5-disubstituted pyridine which is also active against *T. brucei (*Duffy *et al*., 2017) and *P. lfalciparum* (DS lab data). MMV688990 is again a reference molecule with antiprotozoal activity against *L. Donovani* (Monge-Maillo and López-Vélez, 2015). MMV687170, MMV687729, MMV687813 and MMV688844 are also anti mycobacterial molecules but no MOA data is available from literature.

A previous attempt to identify Malaria Box delayed-death inhibitors of *P. falciparum* identified MMV008138, which was found to target the 2-C-methyl-Derythritol 4-phosphate cytidylyl - transferase (IspD) enzyme required for isoprenoid biosynthesis in the apicoplast (Wu *et al*., 2015, Yao *et al*., 2015, Imlay *et al*., 2015). MMV008138, however, exhibits no immediate-killing or delayed-death effects on *T. gondii*. It is worth noting that although *P. falciparum* undergoes delayed death, its association with a defective plastid segregation

phenotype can vary markedly (Dahl and Rosenthal, 2007, Goodman*et al*., 2007). Many of these MMV molecules that target the apicoplast are chemically distinct from the macrolide, tetracycline, lincosamide, and fluoroquinolone class of compounds that are known to cause plastid mis-segregation and delayed death in *T. gondii*. There are some reference molecules known to cause parasite delayed death, which are included in Pathogen Box, such as Levofloxacin, Radezolid, Sutezolid, and Doxycycline, which have shown apicoplast mis-segegation in our screen. This as an indirect validation of our screening strategy and experimental protocols used in this work. Thus, it will be interesting to study the mechanism by which these molecules cause delayed death in *T. gondii*. For the remaining molecules, which cause delayed death but have no apparent phenotypic effect on the plastids in our assays, it needs to be investigated whether they act by targeting plastid-associated housekeeping functions or act via completely different pathways.

Egress of intracellular parasite from host cell is accompanied by host cell cytolysis, involving the concerted action of pore-forming proteins, kinases, proteases, and osmotic factors to compromise the host membrane and trigger catabolic enzymes to eventually dismantle the infected cell (Blackman, 2008). In the case of *P. falciparum*, molecules targeting the cysteine and serine type proteases have differential inhibitory effects on the host RBC membrane and PV membrane, respectively (Hale *et al*., 2017, Rosenthal, 2011), resulting in readily distinguishable microscopic features. Some of these effects are phenocopied in *T. gondii* as well (Roiko and Carruthers, 2009, Kafsack *et al.*, 2009). Our collaborators identified 26 molecules from the Malaria Box collection and 17 from Pathogen Box collection that inhibited the egress of *P. falciparum* merozoites from iRBCs with low micro molar to nano molar potency. Many of these molecules were found to have a comparable inhibitory effect on drug-resistant strains of *P. falciparum* as well.

These assays could be converted to high throughput screen and could be extend to study other aspects of cell biology of parasite and host–parasite interaction where high throughput imaging approaches might be useful. The identification of > 20 delayed death inhibitors and 10 egress inhibitors from the MMV compiled antiparasitic molecules, with comparable phenotypic effects on two different evolutionarily related parasites, provides an avenue to probe conserved targets that could be explored for biochemical and genetic validation in the future. Eventually some of these molecules have the potential to progress as lead molecules for antimalarial or anti-toxoplasmosis therapy.

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Chapter 4

Mechanism of action (MOA) studies and target identification for selected inhibitors.

Introduction

There are various approaches to identify target of a small molecule and each of them have some advantages and drawbacks associated with the technique. For example binding affinity based chemical probes were successfully used to fish out the target for some inhibitors (mostly protease inhibitors) (Carey KL *et al*., 2004, Chandramohanadas *et al*., 2009), but this is not a technique that is universally applicable to all inhibitors. The inhibitor should be amenable for chemical modification, while retaining its potency and specificity. A powerful approach to identify target, that does not require any structural modification of the small molecule inhibitor, is to generate mutants which are resistant to the inhibitor of interest using, and then mapping the mutations associated with the resistance to the target gen(s).In this case, mutation can be induce by natural evolution – by exposing the parasites to sub-optimal dose of the inhibitor or by using a chemical mutagen that will induce random mutations in the genome. Both of these approaches have been successfully used to discover the MOA for novel antiparasitic compounds (Pfefferkorn *et al*., 1992, Camps *et al*., 2002, Amberg-Johnson K *et al*., 2017).

Regardless of the approach, the resistance conferring mutations need to be identified and mapped to the phenotype. Whole genome sequencing of the parent line and isogenic mutant isolate can help in identifying the target genes and mutation that is responsible for resistant development. Recent advancements in whole genome sequencing and reduction in cost have allowed this to be the method of choice for mechanistic studies on novel inhibitory compounds .Validation studies typically involve the introduction of the mutant gene into wild type parasite followed by demonstrating the loss of sensitivity to the inhibitor.

However, this approach may not work if the parasites fail to develop resistance and simply die or have decreased fitness in sub-potent doses of the inhibitor. It is also likely that

the mutations leading to the development of resistance occur in a non-target gene, which simply facilitates inhibitor access to the target. Examples of this include transporters needed to facilitate entry of the inhibitor into the cell or subcellular compartment, and conversion of the prodrug to its active form by cellular metabolism. Therefore, further biochemical and molecular validation is required to prove that the identified mutation is the one responsible for the observed resistance and the associated gene is the actual and only target for the small molecule inhibitor. This approach has proven successful in *T. gondii*, where chemical mutagenesis was used to isolate parasites resistant to the egress and death-inducing effects of the calcium ionophore A23187 (Black *et al*., 2000) and validate parasite actin and tubulin as the targets for cytochalasin D (Dobrowolski *et al*., 1996) and oryzalin (Morrissette *et al*., 2004) respectively.

This Chapter of the thesis elaborates the mechanistic and target identification studies we have carried out for selected MMV Box molecules which target the apicoplast functions in *T. gondii* and impart a delayed death phenotype.

Material and Methods

Estimating the minimum inhibitory concentration (MIC) for inhibition of plaque formation by T. gondii: These experiments were done to estimate the MIC for delayed death inhibitors, so that mutant parasites can be tested in that concentration for loss of sensitivity to the inhibitor. Plaque formation was essential carried out as described in Chapters-2 & -3. HFF host cells were first grown to confluence in six well culture plates, and three such plates were used to find the MIC for each molecule of interest. The inhibitors were used at various concentrations after a two-fold serial dilution starting from 20µM to 0.63µM. RH-*wt* parasites were harvested from infected host cells by gently scraping the infected monolayer with a scraper followed by passing this suspension through 25 gauge needle and filtering it

with 3µm filter membrane. Parasites were counted, diluted and added to these plates at 50 parasites per well. Plates were kept undisturbed in incubators and taken out after 10 days. The monolayer were fixed and stained with crystal violet solution and images for each well were taken using a Canon camera over a white light box. The inhibitor concentration, at which no plaque formation was seen, was considered as the MIC for that molecule. The MIC was determined for 13 molecules prioritized from Chapter-3 studies (**Table-9**).

N-ethyl-N-nitroso urea (ENU) mutagenesis protocol for generating resistant mutant generation:

Preparation of ENU stock: A 1M stock of ENU (Sigma-Aldrich) was prepared in DMSO. This solution was distributed in multiple aliquots of 1ml each and was stored in deep freezer. For each experiment a new vial was thawed out and final working stock was prepared in complete parasite medium.

Preparation of ENU decontamination solution: A10% sodium thiosulfate solution was prepared fresh in water and was used as decontamination solution during the experiments. The spent and leftover ENU containing solutions were mixed with the decontaminating solution before disposal as per chemical safety regulations.

Standardization of ENU concentration: Freshly isolated tachyzoite stage *T. gondii* were washed and resuspended in complete parasite culture media.$1x10^8$parasites were added per T-25 flask containing confluent HFF monolayer. Parasites were allowed to infect and replicate within host cells for 24 h in normal culture media. Then the culture medium was replaced with 1mM and 2mM ENU containing medium. This treatment was done in triplicates. ENU untreated parasites were washed and treated with the same concentration of vehicle (DMSO) only, again in triplicates.

ENU treatment was done for 2 h under optimal culture conditions. Utmost care need to be taken during these experiments and caps of the flasks should be tighten up properly to avoid any accidental spilling of the mutagen. After 2 h, the spent medium was removed and dispensed in the decontaminating solution (10% sodium thiosulfate). All flasks were washed thrice with pre-cooled complete medium followed by harvesting the mutated parasites from infected host cell monolayer. Parasites were counted and used for plaque forming assay to estimate the viability of the parasite. For this, 150 parasites from each treatment set were inoculated into a fresh flask containing confluent monolayer of HFF cells. These flasks were incubated at 37°C in a humidified incubator maintaining 5% CO_2 for 10 days, after which the infected monolayer was fixed with methanol and stained with crystal violet to visualize plaque formation. Plaques were counted in each flasks and data was plotted to determine the percentage survival of ENU treated parasites, in comparison to untreated parasites.

Selecting ENU mutagenized parasite population with the inhibitors of interest: This was done to select out the parasites, which have become resistant to the inhibitor, from other parasites with unwanted mutations. For this, ENU mutagenesis (as described above) was done in three independent culture flasks, and the mutated parasites isolated from each flask were separately resuspended in fresh culture media. Selection of the three ENU mutagenized parasite populations with the inhibitor molecule was done independently by inoculating the total parasites isolated from each ENU treated flask into a separate T-175 flask.

The cultures were then incubated for 24 h under optimal conditions to support the growth of the mutant parasites in normal culture medium. After this 24 h period, the culture medium was replaced with media containing the inhibitor of interest at the MIC levels, which showed inhibition of plaque formation by wild type parasites. These flasks were left undisturbed for 10 days, following which they were visually analyzed for plaque formation. The presence of a plaque will suggest that the parasites are able to tolerate the inhibitor at

MIC levels. These parasites were harvested and inoculated into a new flask in the presence of inhibitor at 2X MIC levels. After each cycle of host monolayer lyses, the parasites were inoculated into a fresh flask and allowed to replicate in the presence of 2X concentration of the inhibitor over that used in the last flask until a maximum concentration of 20 µM was reached. In the event that the mutant parasites stopped growing and died at an inhibitor concentration less than 20 µM, then further assays were carried out at the maximum tolerable concentration of the inhibitor. After the final round of selection with the inhibitor, the mutant parasites were cloned out by the limiting dilution method and the clonal lines were used for all further studies.

Isolation of genomic DNA from mutant parasites and identification of the mutations responsible for resistance by whole genome sequencing:

Genomic DNA was isolated from the desired parasite lines using the genomic DNA isolation kit (Qiagen) following manufacturers protocol. Following DNA extraction, the genomic DNA for each sample was quantitated using the Qubit® 2.0 Fluorometer and was used for genome sequencing library preparation. The extracted DNA was sheared using a Covaris E220 DNA sonicator to fragments of 500bp. The DNA libraries of interest were made using the NEBNext Ultra II DNA Library Prep Kit (NEB) (Nebnext) according to the manufacturers' instructions. The amplified libraries were stored in -20 °C. The pooled libraries were sequenced in an Illumina HiSeq4000 instrument at KAUST Core Lab facility generating approximately 10-15 GB of sequencing data per sample. A PhiX control library was applied to the sequencing run as a base balanced sequence for the calibration of the instrument so that each base type is captured during the entire run. Raw sequence reads were submitted to FastQC v.0.11.5 and the quality score of the sequences generated was determined.

***Strategy for identifying the gene specific mutations conferring resistance to the inhibitor*:** Analysis of the ENU induced single nucleotide polymorphisms (SNPs) and INDELs (insertions and deletions) present in the genome of the mutant parasites can help in the identification of the genes, which code for the target proteins of the inhibitors. The genome sequence data obtained for the various mutant parasites isolated in this study contained many SNPs and INDELs. This is expected since ENU treatment induces the formation of multiple random mutations per genome. Therefore we formulated a set of criteria for selecting the correct gene(s) to validate for the resistance phenotype. These criteria are listed below –

1. The mutations found in common with the parental strain of the parasite were ignored.
2. Only mutations found in the gene-coding regions that resulted in a non-synonymous change (i.e., amino acid change) were considered.
3. Mutations that resulted in complete loss of function (either by shifting the coding frame or by truncating the gene product) were disregarded. This criterion was formulated based on the reasoning that a complete loss of target gene function would be similar to the inhibitory effect of the compound and therefore should result in parasite death. The fact that the resistant parasites are alive suggests that the target gene function is intact.
4. Only mutations in genes that are expressed in the tachyzoite stage were considered.
5. Finally, only mutations occurring in essential genes (identified previously by a CRISPR/Cas9 screen (Sidik *et al*; 2017)) were considered.

Only the mutations qualifying for the above mention criteria were considered to select the genes for target validation experiments.

Confirming the mutations identified from genome sequence data by resequencing gene specific PCR amplicons: After selecting and prioritizing the target genes as mentioned

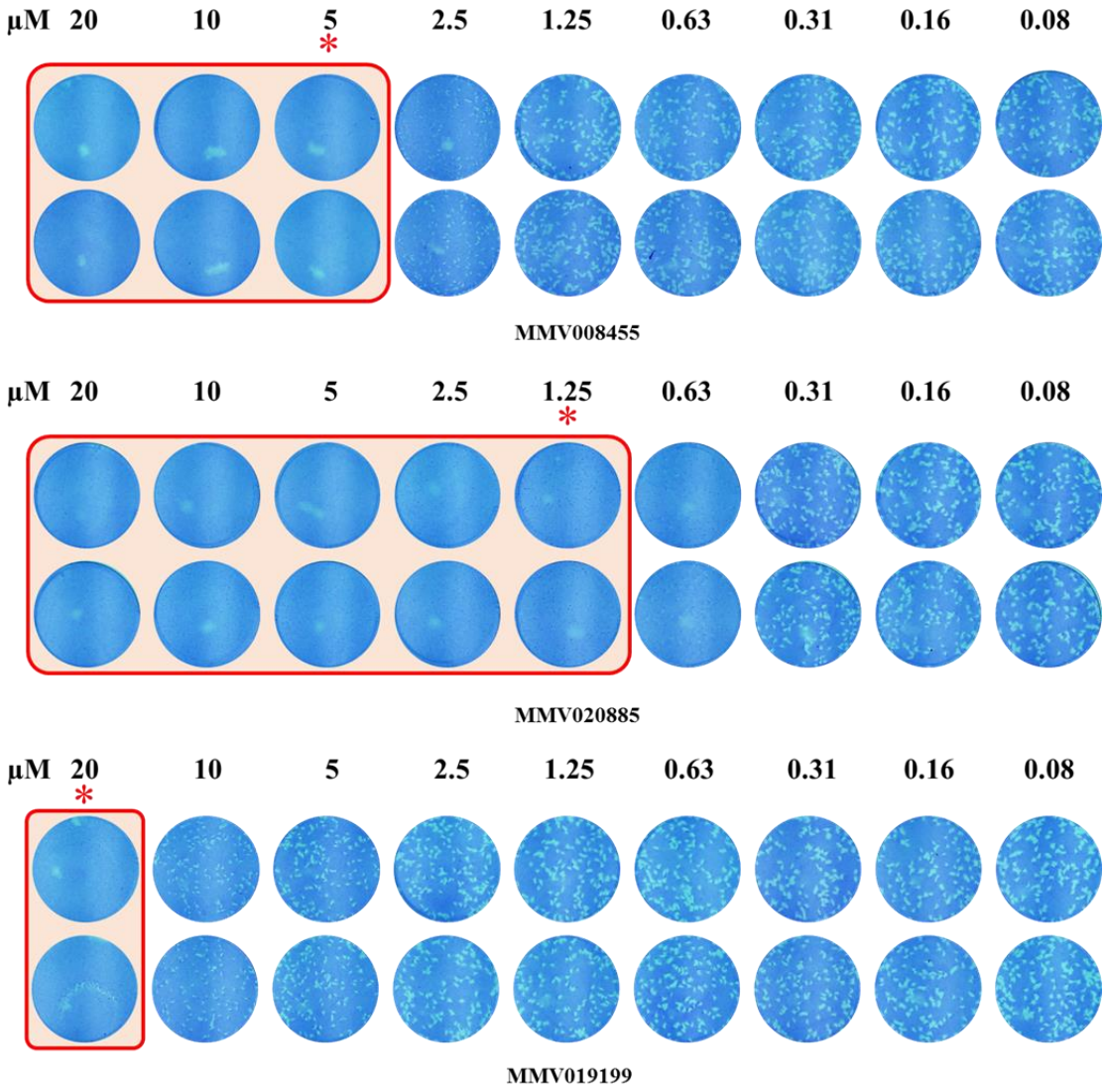
above, the presence of the mutation was confirmed by PCR amplifying the relevant gene fragment and resequencing by Sanger method. For this, total RNA was isolated from the resistant mutants and parental line, and cDNA was prepared and used as template for PCR amplification of the corresponding full-length cDNA. The amplicons were cloned into the pGEM-T easy plasmid (Promega) and sequenced by Sanger method to confirm the presence of the mutations.

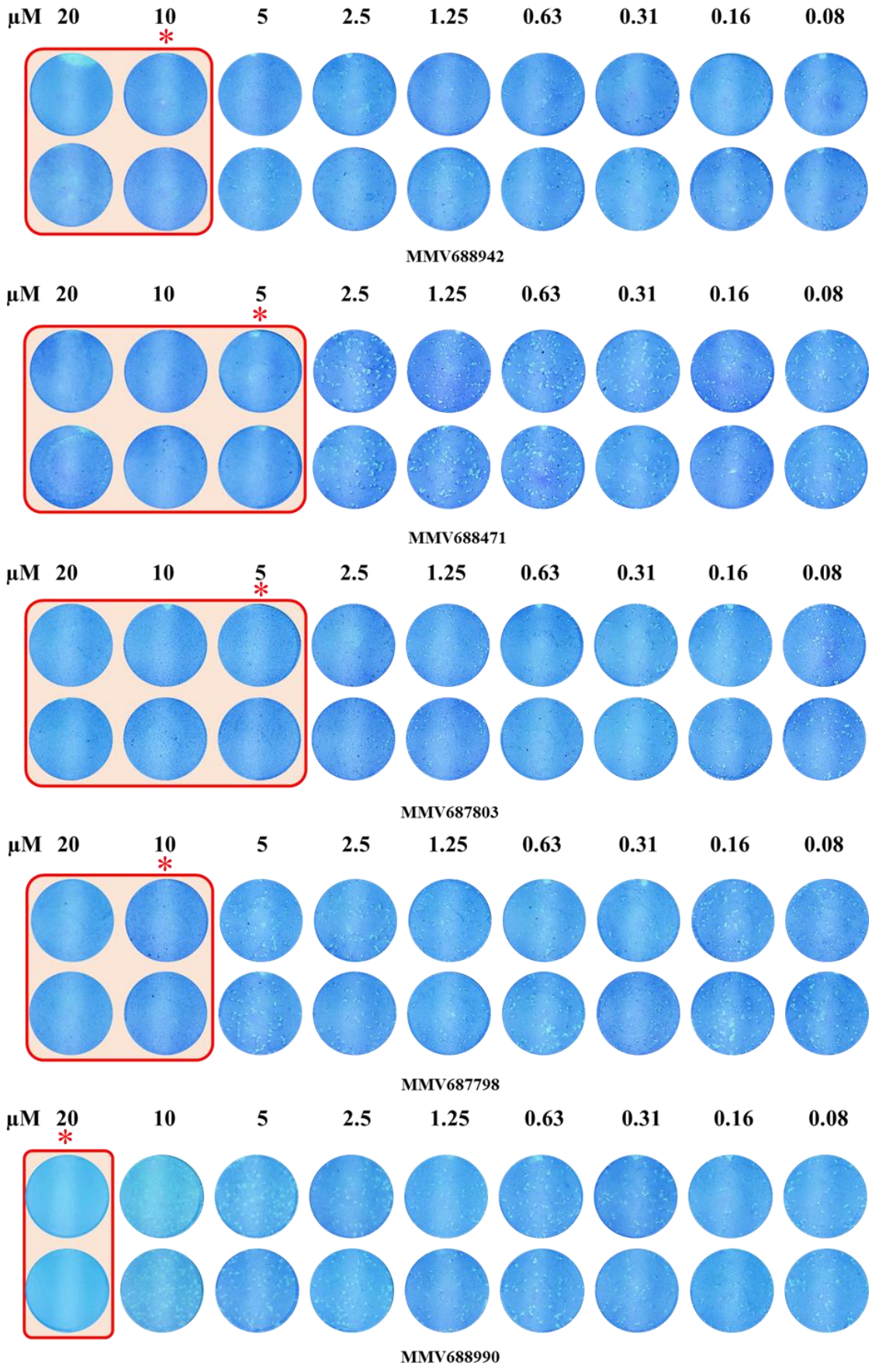
Target validation by expressing the mutant gene in the wild type parasite and demonstrating loss of sensitivity to inhibitor: The full-length cDNA of the mutated gene PCR amplified from the resistant mutant was cloned into the *T. gondii* expression plasmid and constitutively expressed with a C-terminal HA epitope tag in wild type tachyzoite stage parasites. For details of the expression plasmid and transfection protocol, see Chapter-2 methods section. Stable transgenic lines expressing the gene of interest were generated using chloramphenicol selection and cloned by limiting dilution method.

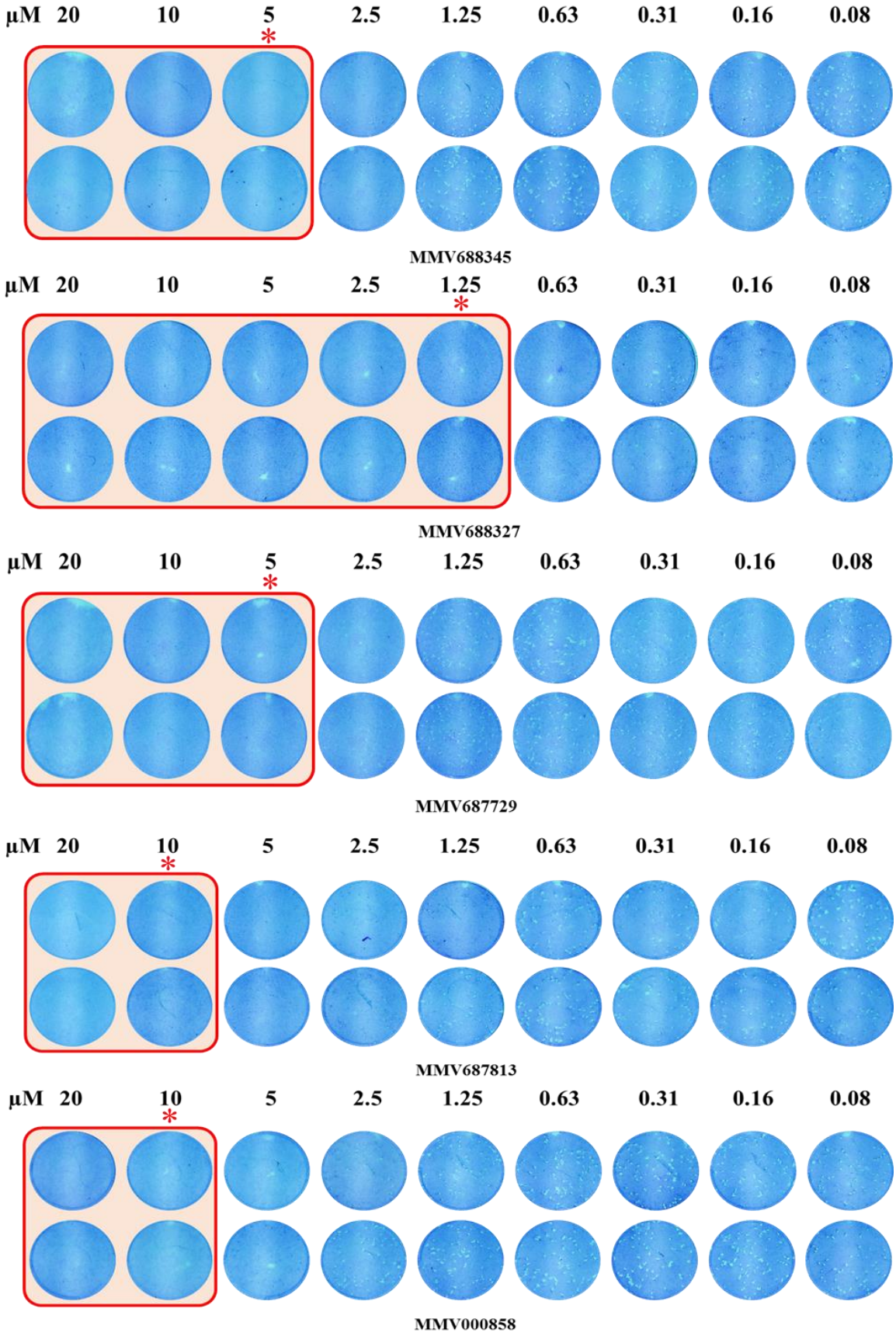
Target validation studies for the MMV688345 inhibitor molecule: Although resistant mutants were isolated for a few different inhibitors, for this thesis work we have pursued the target validations experiments for one of the inhibitors from the MMV Box, MMV688345. Based on the genome sequence data and gene selection criteria detailed above, we were able to narrow down the *Tgdhfr-ts* gene as the likely target for this inhibitor. To validate the *Tgdhfr-ts* gene as the *bona fide* target for MMV688345, we cloned the full-length mutant and wild type genes and expressed the two genes constitutively in wild type parasites from an expression plasmid. Transgenic wild type parasites expressing either of the *Tgdhfr-ts* genes were evaluated for delayed death by plaque forming assays in the presence and absence of the MMV688345 inhibitor at 20µM and further serial two fold dilutions. Plaque forming assays were carried out as described in Chapters-2 & -3.

Results

Identification of MIC levels for plaque formation of selected delayed death causing molecules: 13 molecules were selected to perform mutagenesis experiment on the basis of consistency of the plastid mis-segregation and delayed death phenotypes. Out of these 10 were Pathogen Box molecules and 3 were Malaria Box molecules. Details of these molecules are described in **Table-9**. Some of these molecules also had previously reported data on likely MOA in other parasites. MIC for inhibiting plaque formation was determined by performing plaque assays for all 13 molecules (**Figure-4.1)**.




µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
MMV688942
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
MMV688471
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
MMV687803
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
MMV687798
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
MMV688990


µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
*
MMV688345
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
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MMV688327
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
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MMV687729
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*
MMV687813
µM 20 10 5 2.5 1.25 0.63 0.31 0.16 0.08
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MMV000858

Figure-4.1: **Determination of MIC for inhibition of plaque formation** for the 13 molecules selected from MMV Box libraries. Each panel shows the plaque formation in duplicate at various concentrations of the inhibitor molecule. The numbers indicate inhibitor concentration in µM. The respective MIC values are highlighted in yellow for each molecule and summarized in **Table-9**.

ENU mutagenesis successfully generated resistant mutants to different inhibitors: ENU, also known as N-ethyl-N-nitrosourea (chemical formula C3H7N3O2), is a highly potent mutagen. ENU is an alkylating agent, and acts by transferring the ethyl group of ENU to nucleobases (usually thymine) in nucleic acids. A chemical mutagenesis method to determine the target and study the MOA of small molecule inhibitors was found to be successful in reported studies. In this study we have used ENU as the chemical mutagen, which is proven to be more effective in case of *T. gondii* mutagenesis studies in comparison to other available mutagens (Farrell *et al*; 2014).

Compound ID	MIC plaque	Common name	Organisms screened	Target if any predicted
MMV688942	10 µM	Bitertanol	Fungi	Sterol Biosynthesis
MMV688471	5 µM	-	Cryptosporidium/Toxoplasma	IMPDH
MMV000858	10 µM	-	Plasmodium/T.gondii/Trypanosoma sp	-
MMV687803	5 µM	-	Plasmodium	Delayed death causing
MMV688327	1.25 µM	-	-	-
MMV687798	10 µM	Iquir/Levofloxacin	-	DNA gyrase
MMV688345	5 µM	-	Pneumocystis/T.gondii	DHFR
MMV687729	5 µM	-	-	-
MMV687813	10 µM	-	-	-
MMV688990	10 µM	Miltex/Miltefisine	Trypanosoma sp. /Leishmania sp	Cycline dependent Kinase
MMV008455	5 µM	-	-	-
MMV020885	1.25 µM	-	-	-
MMV019199	10 µM	-	-	-

Table-9: **A list of the 13 molecules selected for ENU mutagenesis:** 13 molecules selected for ENU mutagenesis along with corresponding plaque inhibitory MIC levels and other details of the molecules.

Standardization of appropriate ENU concentration to use for generating the mutant parasites: ENU causes random mutagenesis across the whole genome and mutations within essential genes will cause parasites to die. In order to minimize the number of mutations per genome and to try and achieve genome wide unbiased mutations, which are non-lethal, we needed to determine the dose and duration of ENU treatment for tachyzoite stage parasites. As per available literature data, ENU can be used at 1M.From our standardization experiments, we also found that following 1mM ENU treatment, tachyzoite stage *T. gondii* had a 50% survival rate (**Figure-4.2**).

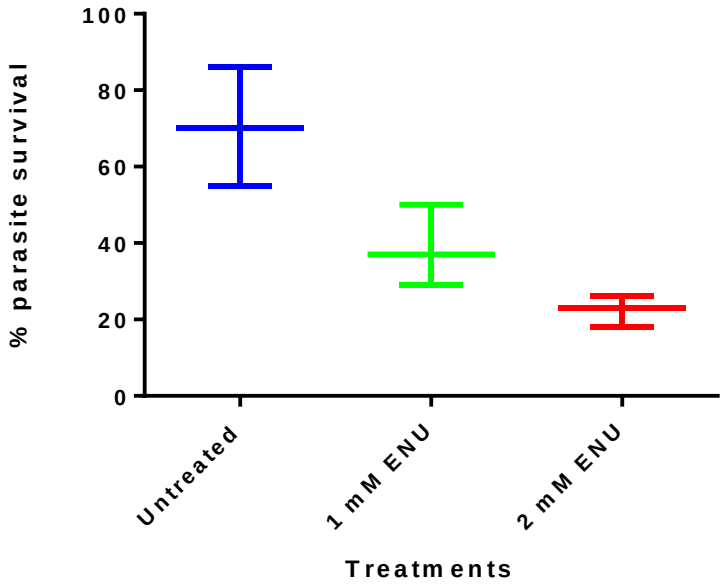

Figure-4.2: **Determination of ENU concentration**: RH -*wt* parasites were used to determine the appropriate ENU concentration to use in the mutagenesis experiments. The concentration at which the median percent survival of the parasite was close to 50% was taken to perform the mutagenesis experiments.

Selecting mutant parasites resistant to selected inhibitors following ENU mutagenesis: To select for resistance, the ENU mutants were treated with the selected molecules at MIC levels, which inhibit plaque formation by wild type parasites. We have followed a selection protocol that is schematically represented in **Figure-4.3**. Out of 13 molecules selected, we were able to get resistant mutants against 7 of them. All of these were capable of tolerating

the inhibitor molecules up to 20µM. We isolated clonal lines for each of these resistant parasites, and three clonal lines for each molecule were taken further for genome sequencing in order to identify the underlying mutation(s) responsible for the resistant phenotype. We double checked each of these clonal isolates again in plaque forming assays to ensure that the phenotype is reproducible before embarking onto genome sequencing.

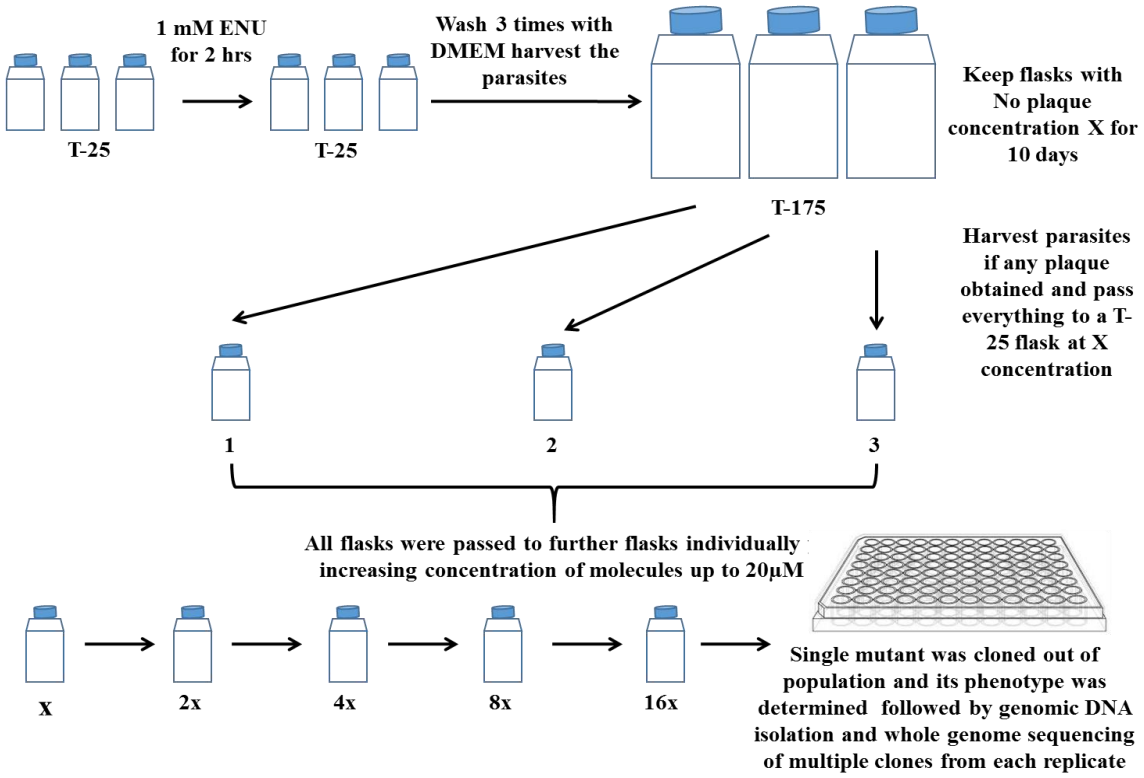

Figure-4.3: Overview of the ENU mutagenesis and inhibitor resistant mutant selection and isolation protocol used in this study.

***Whole genome sequencing of parent and mutant lines of the parasite*:** Wild type parental parasites and 5 different mutant parasites were sequenced. Three different clonal lines of the wild type parasites were sequenced and compared to the reference genome available in *ToxoDB.org*. For three of the mutants, three different clonal lines were sequenced, while for the other two mutants, two clonal lines and one clonal line respectively, were sequenced. On average, 60X coverage was achieved for all the sequenced genomes and > 95% of the

genome was represented in the sequence data for all samples. SNPs and INDELs were detected by comparing the sequenced genomes to the *T. gondii* ME49 strain reference genome available from *ToxoDB.org*. SNPs were called using GATK pipeline. SNP and INDEL sites differing from wild type genotypes were filtered and checked if they were present on all replicates. High Impact mutations, which confer non synonymous changes and can potentially disrupt the function of the gene, were predicted by SNPeff tool. The numbers of genes affected by high impact SNPs and INDELs, in all 5 mutant lines, were compiled and are listed in the **Table-10**.

Compound	replicates	total SNPs and indels	High impact SNPs and indels	number of genes affected
MMV688471	3	1479	114	53
MMV019199	3	1897	173	60
MMV008455	3	2364	164	78
MMV688345	2	4611	409	128
MMV020885	1	5335	356	124

Table-10: List of molecules for which resistant mutants were generated by ENU mutagenesis and compilation of the consensus high impact SNPs and INDELs identified by sequencing the genome of these mutants.

Identifying specific mutations in genes responsible for the resistance phenotype: By following the checklist of criteria listed in the methods section for selecting the most likely target gene, we have prioritized two of the mutants, one was resistant to MMV688345 and the other was resistant to MMV688471. Details of the selection criteria, for these molecules, are shown in **Table-11**. These were the two mutants for which we could unambiguously narrow down a few probable candidate target genes, which can be validated further. We decided to pursue validation experiments for the MMV688345 molecule first as there is significant published literature on the likely MOA of this molecule.

Compound	Number of genes affected	Non-specific genes	Specific genes	Expressed in tachyzoite stage*	Mutation resulting in gene disruption	CRISPR/Cas9 Essentiality#	Selected for Validation
MMV688471	53	51	2	2	0	2	**2**
MMV019199	60	52	8	6	0	4	n/a
MMV008455	78	56	4	3	1	2	n/a
MMV688345	128	100	34	25	4	9	**5**

Table-11: **Compilation of the number of genes affected by ENU mutagenesis** for 4 MMV Box molecules. The different criteria used for shortlisting the genes is given and numbers indicate the total number of genes qualifying progressively from left to right. The two molecules prioritized for validation studies are sown in red.

Validation of resistant phenotype by plaque forming assays: After isolating clonal lines of the mutant parasites, we have determined their plaque forming ability in the presence and absence of the concerned inhibitor in comparison to the parental lines of the parasite. Since we have prioritized the mutants resistant to the MMV688345 inhibitor for validation studies first, from here on all experimental data discussed will be focused on this molecule and its corresponding resistant mutant. The MIC for inhibition of plaque formation by wild type parasites was 5µM for MMV688345. In case of the mutant parasite, the MIC value for MMV688345 was found to be 20µM (**Figure-4.4**).

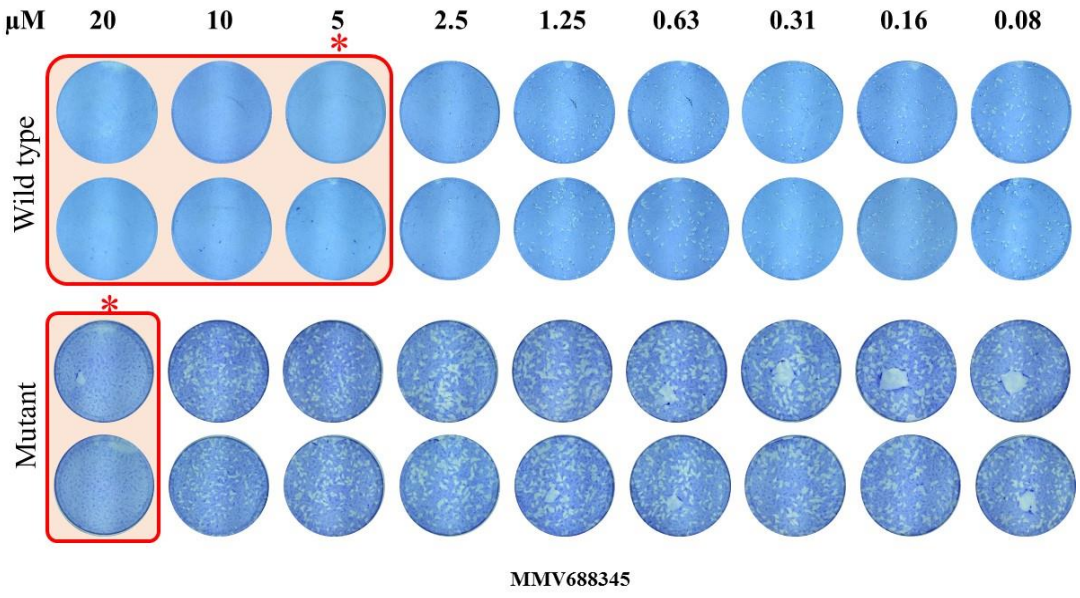

Figure-4.4: Determining the MIC value for inhibition of plaque formation in wild type and in mutant parasites showing resistance to MMV688345. The various concentrations of the inhibitor tested are shown in µM values above the panel.*, Denotes the MIC value.

Identifying the TgDHFR-TS enzyme as the target of MMV688345: The mutants with resistance to MMV688345 isolated from the ENU mutagenesis experiment consistently showed a single base pair change in *Tg*DHFR-TS gene. This mutation imparts a phenylalanine to leucine change at position 32, which maps to the DHFR domain of the enzyme (**Figure-4.5**). The IUPAC name of MMV688345 is 4-(2, 5-Dimethoxyphenyl)-3-buten-2-one, and this molecule was previously shown to inhibit the recombinant *Tg*DHFR-TS enzyme (Rosowsky *et al*; 1994). *Tg*DHFR-TS is a bifunctional enzyme and plays an important role in nucleotide biosynthesis and hence a valid drug target against *T. gondii*. From multiple sequence alignments of DHFR-TS protein from some apicomplexan species, we find that the phenylalanine residue is conserved across all coccidian parasites, but is methionine in case of sporozoa (**Figure-4.5**). *Cryptosporidium* naturally has a leucine at this position, which suggests that the mutation in *T. gondii* will not make the DHFR-TS enzyme inactive. However, the mutation may be effective in only mitigating the effect of the inhibitor on the enzyme by likely decreasing its binding affinity to *Tg*DHFR-TS.

It is interesting that inhibition of *Tg*DHFR-TS by MMV688345 leads to delayed death of the parasite, since inhibition of the enzyme by pyrimethamine results in immediate death of the parasite. We therefore tested the effect of pyrimethamine of MMV688345 resistant mutants. We carried out the plaque forming assays to determine the sensitivity of mutant parasites towards pyrimethamine and MMV688345, and surprisingly found that the mutant parasites showed partial resistance towards pyrimethamine (**Figure-4.6**). Although multiple other mutations in the *Tg*DHFR-TS have been shown to protect against pyrimethamine, the F32L mutation identified in this study has not been reported before.

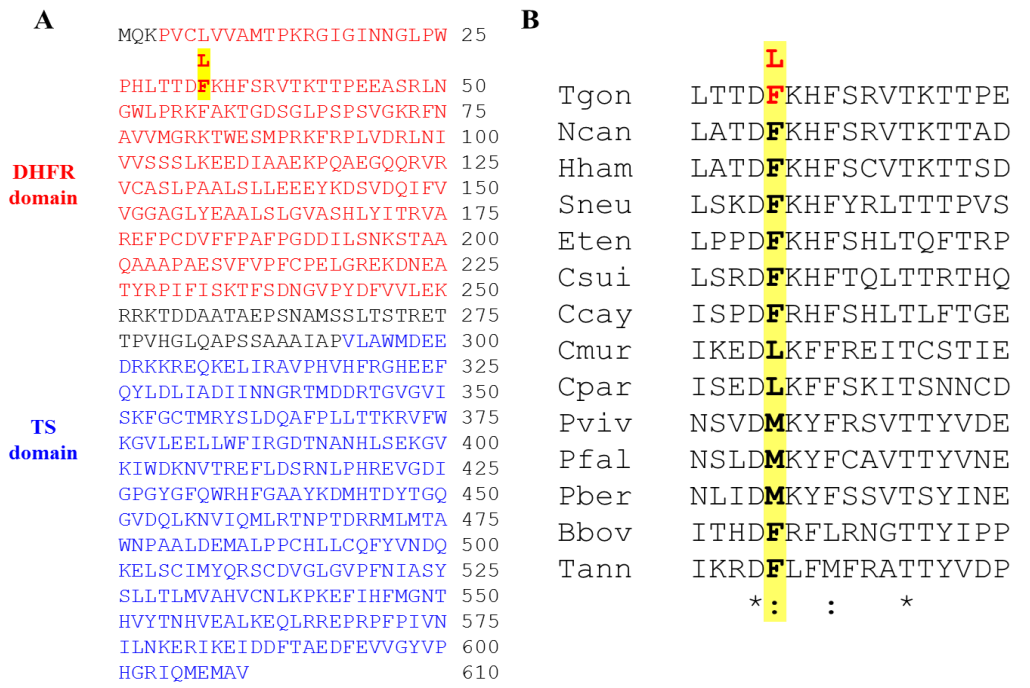

Figure-4.5: Mapping the mutation observed in the mutant parasite to the N-terminal domain of *Tg*DHFR-TS enzyme. A) Mutation at amino acid position 32 changes phenylalanine to leucine in the DHFR domain of the enzyme. B) Multiple sequence alignment of representative apicomplexan parasites. The position corresponding to amino acid mutation in *T. gondii* protein is highlighted in yellow. Species names: Tgon, *T. gondii*; Ncan, *Neospora caninum*; Hham, *Hammondia hammondi*; Sneu, *Sarcocystis neurona*; Eten, *Eimeria tenella*; Csui, *Cystoisospora suis*; Ccay, *Cyclospora cayetanensis*; Cmur, *Cryptosporidium muris*; Cpar, *C. parvum*; Pviv, *P. vivax*; Pfal, *P. falciparum*; Pber, *P. berghei*; Bbov, *Babesia bovis*; Tann, *Theileria annulata*.

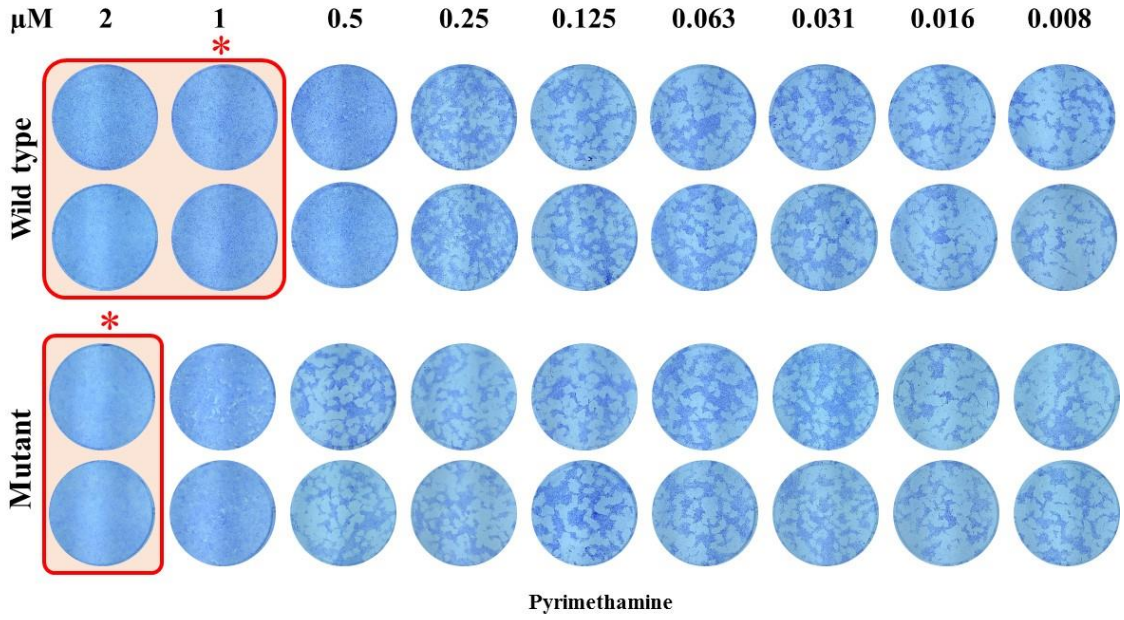

Figure-4.6: Plaque forming assays, showing the partial resistance of the mutant parasites toward pyrimethamine. The MIC for pyrimethamine in wild type parasites is 1 µM while in the F32L mutant it has shifted to 2 µM.*, Denotes the MIC value.

Genetic validation of TgDHFR-TS as the target of MMV688345: In order to validate that *Tg*DHFR-TS enzyme is the *bona fide* target for MMV688345 inhibitor, we decided to ectopically express the cDNA coding for the mutant *Tg*dhfr^{F32L} in wild type parasites and check the delayed death effect of MMV688345 on these transgenic parasites. Our expectation was that if the expression of the mutant DHFR-TS enzyme in wild type parasite will make these parasites resistant to MMV688345, it will be a straight forward way to validate the inhibitor-target relationship. We therefore cloned the full-length cDNA for *Tg*dhfr^{F32L} into the *T. gondii* expression, which will express the gene constitutively under the β-tubulin promoter.

Since, in addition to the expression of the mutant enzyme, the wild type enzyme will also be expressed in these parasites, it will result in increased activity of the enzyme in the transgenic parasite. To control for the expression level bias, and to rule out unintended resistance to the inhibitor arising due to over-expression of the enzyme, we also generated transgenic parasite lines in which the wild type enzyme was constitutively expressed from the same expression plasmid used for the mutant enzyme.

Clonal isolates of two stable lines of transgenic parasites, one expressing the mutant *Tg*dhfr^{F32L} enzyme and the other expressing the wild type enzyme were simultaneously tested for plaque formation in the presence of the inhibitor. We found that the parasite expressing the mutant enzyme showed similar resistance phenotype to that observed with ENU mutant line (**Figure-4.7**). However, parasites expressing the wild type enzyme remained sensitive to the inhibitor with an MIC value of 5 µM, thereby validating *Tg*DHFR-TS as the target for MMV688345.

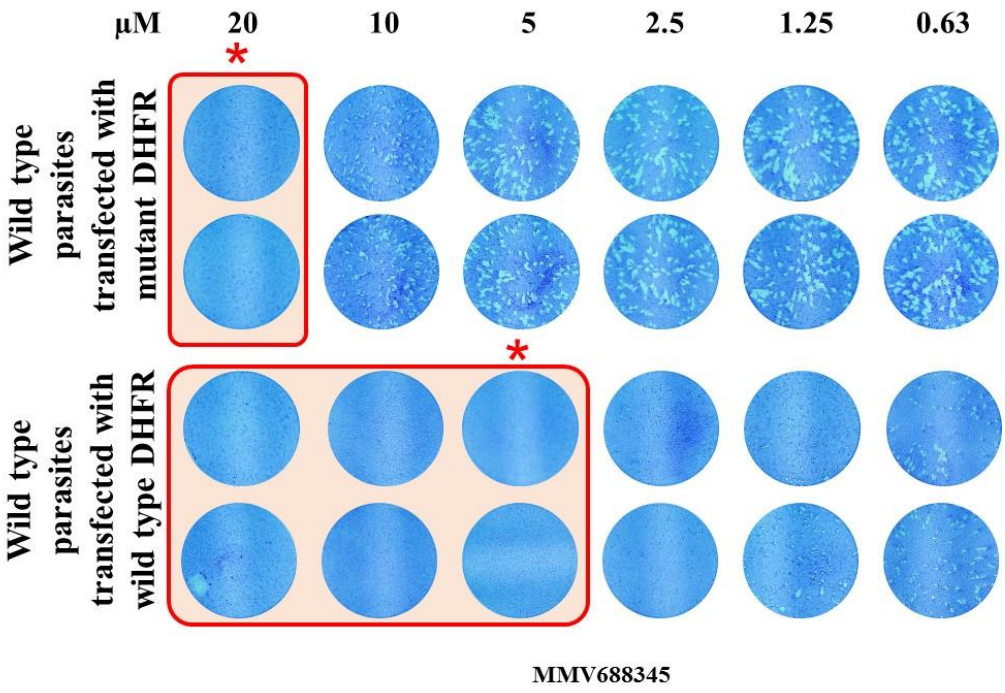

Figure-4.7: **Plaque assays were performed to validate *Tg*DHFR-TS as the target for MMV688345**. Wild type parasites expressing the mutant enzyme (top panel) show a higher MIC value of 20 µM. Wild type parasites expressing the wild type enzyme (bottom panel) showed a similar MIC value of 5 µM as seen with normal wild type parasites. The numbers on top show the concentration of MMV688345 used in µM. *, Denotes the MIC value for inhibition of plaque formation.

Discussion and Conclusion

Apicomplexan parasites are an extremely diverse group of obligate intracellular protozoan parasites. A shared hallmark of these parasites is the presence of the apical end organelle complex. Another important cellular feature, in some but not all, of these parasites in the presence of the apicoplast organelle, an unusual plastid organelle with a secondary endosymbiotic ancestry. *Toxoplasma*, being an ideal member of this group for studying cellular processes, also provides a convenient system for genetics, which is lacking in most other apicomplexan parasites. Surprisingly, these parasites are sensitive to inhibitors of the prokaryotic trans-peptidation process, such as macrolides, chloramphenicol, tetracyclines, and lincosamides (Fichera *et al*., 1997). These antibiotics are quite promising as alternative

therapeutic options for the management of various diseases caused by these parasites.infection, because the available and standard therapies often have problems of toxicity or of resistance development. Direct genetic evidence suggests that they target apicoplast housekeeping functions (Camps *et al*., 2002).

These molecules exhibit peculiar killing kinetics; their inhibitory effect on the parasites is not quick but delayed by at least one round of intra-vacuolar growth and replication. This slow growth inhibition is referred to as delayed death and can be observed as a specific phenotype involving apicoplast mis-segregation during daughter cell formation. This results in a parasitophorous vacuole containing a mixture of plastid-bearing and plastid-less parasites. From this thesis related studies, we have identified around 27 molecules from phenotypic screens, which cause apicoplast mis-segregation in *T. gondii*. To gain insights into the mechanism of action of few of these molecules, we employed a chemical-genetics approach, in which chemically mutagenized parasites were selected for resistance to the inhibitors of interest. Here, we used the chemical mutagen N-nitroso-N-ethylurea (ENU), an agent used successfully in earlier reports to generate useful mutant phenotypes in *T. gondii* (Pfefferkorn *et al*., 1976, Radke *et al*., 2000, Uyetake *et al*., 2001).

Using this method, we were able to generate resistant mutants for 7 MMV Box molecules and focused on the target identification of one molecule, which is MMV688345. This molecule is a methotrexate derivative and was reported to be active against *T. gondii* DHFR enzyme in biochemical assays (Rosowsky *et al*., 1994). In our screens, this molecule was found to cause delayed death of tachyzoite stage *T. gondii*, and we could also observe the accompanying plastid mis-segregation phenotype. The parasite DHFR-TS enzyme plays an important role in folate cycle where it converts dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the *de novo* synthesis of purines, thymidylic acid, and certain amino acids (**Figure-4.8**).DHFR has a critical role in regulating the amount of

tetrahydrofolate in the cell. Tetrahydrofolate and its derivatives are essential for purine and thymidylate synthesis, which are important for cell proliferation and cell growth.

In *T. gondii* this enzyme is a bifunctional enzyme which is known as DHFR-TS and its fine tuning is very important for the functioning of both the enzymes. Antifolates, such as pyrimethamine, are fast killers of *T. gondii*. Thus the delayed death effect mediated by a DHFR inhibitor was a surprising finding. Unfortunately our primary screens were carried out with parasites that were already resistant to pyrimethamine, by virtue of expressing a mutant form of the DHFR-TS enzyme. Therefore, to ascertain that the phenotypic effects observed for MMV6883345 were not due to some effect from the mutant DHFR transgene, we have conducted all our validation experiments with MMV6883345 on wild type parasites.

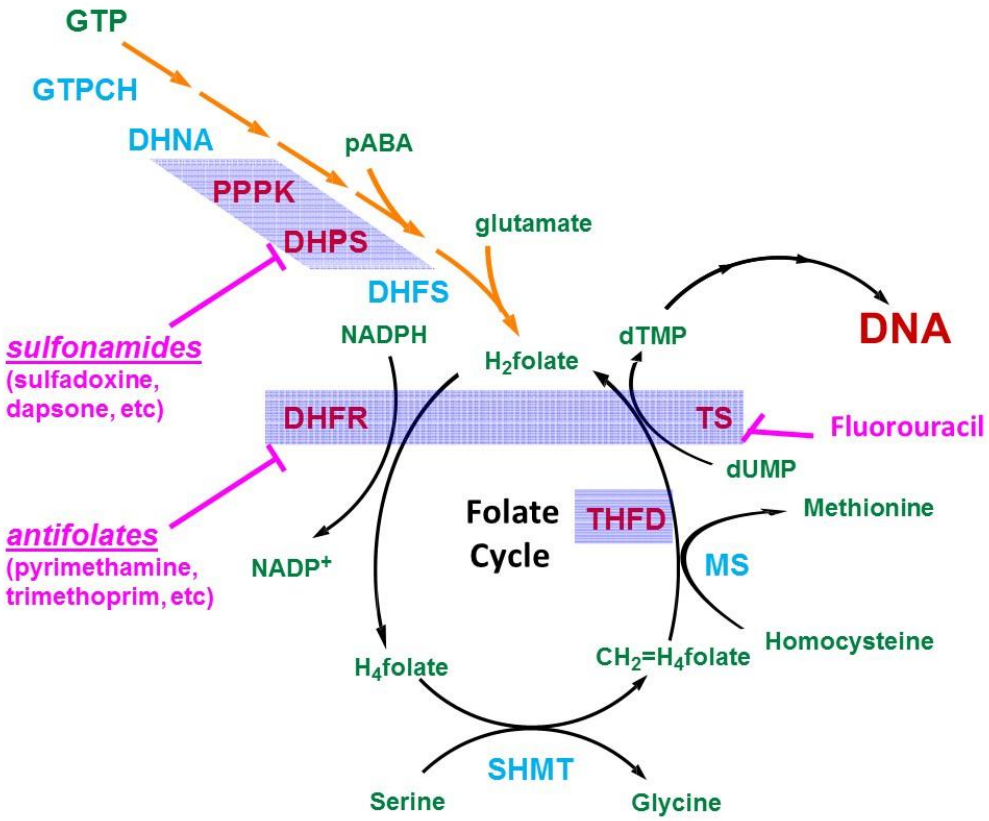

Figure- 4.8: **Schematic diagram of folate cycle in *T. gondii* along with known inhibitors of the pathway**.

These experiments once again confirmed that MMV6883345 induced delayed death of *T. gondii*.

Moreover, in addition to being resistant to MMV688345, the mutant parasites developed partial resistance towards pyrimethamine as well. Based on mutation mapping studies, we identified that the mutant enzyme bears a single amino acid change at position 32 (F32L), which confers resistance to both MMV6883345 and pyrimethamine. This amino acid was shown to be important for methotrexate interaction in earlier reports (Sharma *et al*; 2013, Welsch *et al*; 2016). Fortunately, *Pf*DHFR-TS structure is available with bound pyrimethamine and NADP (**Figure-4.9**). Interestingly, the mutation responsible for resistance toward MMV6883345 (F32L) appears to be in a loops region that is away from the pyrimethamine and substrate binding sites (**Figure-4.9**). It is still not clear how this mutation confers resistance to pyrimethamine. Currently, we are further testing this by generating resistant mutants against pyrimethamine and cross-checking their sensitivity toward MMV688345.

We have also carried out genetic complementation studies to validate that the *Tg*DHFR-TS enzyme is the *bona fide* target for MMV688345. We cloned and expressed the mutant *Tg*DHFR-TS^{F32L} gene in the wild type genetic background and showed that the presence of the mutant enzyme alone is sufficient to confer resistance to MMV688345. The reason for delayed death of parasites treated with MMV688345 is still not clear. We hypothesize that in the presence of a potent inhibitor of DHFR, like pyrimethamine, the function of the DHFR enzyme is completely inhibited. This results in dramatic metabolic effects, such as a complete block of the 1 C cycle, leading to immediate death of the parasite. However, when a weak inhibitor, such as MMV688345 is present, the function of the enzyme is only partially inhibited.

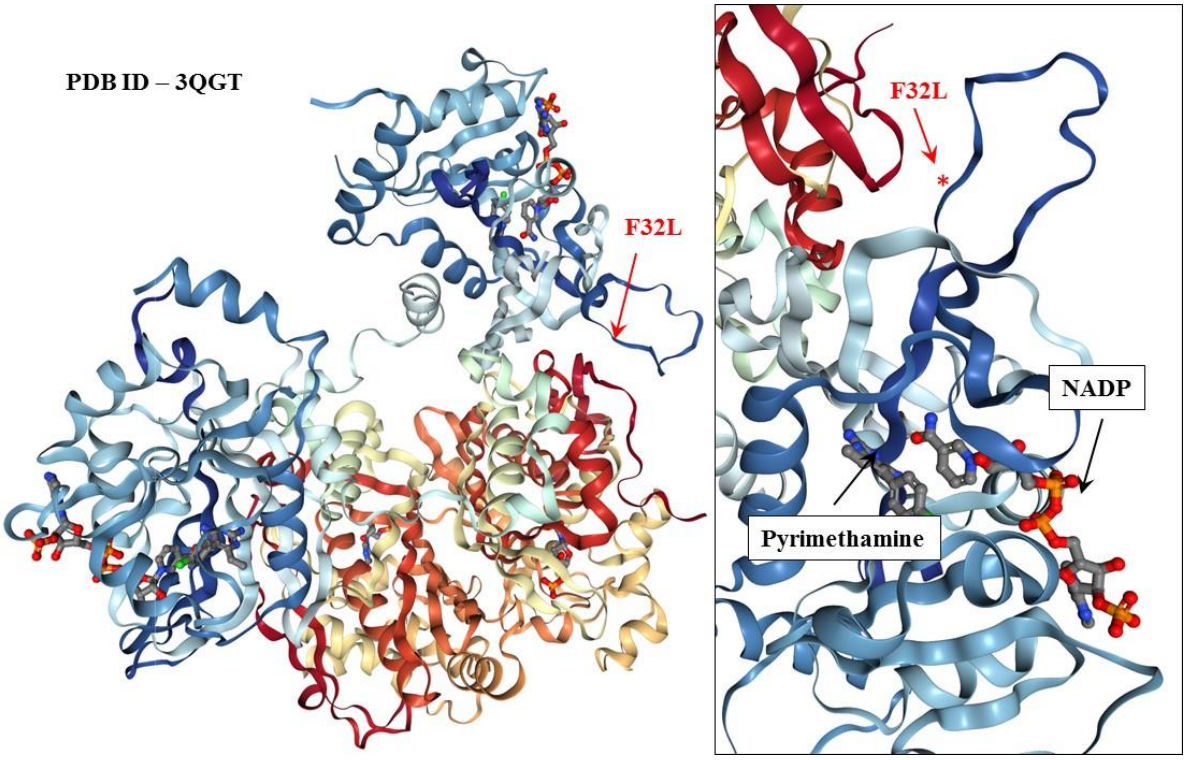

Figure-4.9: **The *P. falciparum* DHFR_TS enzyme structure with bound pyrimethamine and NADP ligands.** The F32L mutation identified in the *T. gondii* mutant is mapped to a loop region highlighted by the red arrow in the left panel and red star in the right panel. Data source: PDB repository.

Due to the reduced efficiency of the enzyme in the presence of MMV688345, DHFR-TS functions at a sub-optimal level leading to a reduction in nucleotide levels within the cell. It is likely that the decrease in nucleotide levels first affects the apicoplast genome maintenance more than the nuclear genome maintenance. Thus, suboptimal functioning of the *Tg*DHFR-TS enzyme may result in inhibition of housekeeping functions in the apicoplast, causing apicoplast mis-segregation phenotype and ultimately delayed death of the parasite. This hypothesis needs to be proven and this would involve other important players like nucleotide transporters on the membrane of cellular compartments like nucleus, apicoplast and mitochondrion.

In summary, this study has opened up new scenarios in parasites biology, which offers the scope for discovering novel antiparasitic molecules. We are in the process of

identifying targets of other mutant parasites, which are resistant to other MMV molecule, and we believe that the insights presented here will pave the way toward forward genetic studies in these parasites. Overall, we have successfully applied a chemical-genetics approach for target identification and MOA studies on promising small molecule inhibitors with anti-plasmodial and anti-toxoplasma activity.

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Chapter 5

Summary of the thesis and future prospects

Introduction

Phylum apicomplexa comprises of thousands of unicellular eukaryotes, which are predominantly obligate intracellular parasites by nature. Morphologically they are distinguished by the presence of a unique set of organelles at the apical end of the cell, necessary for host cell invasion and formation of a parasitophorus vacuole within which the parasites reside. A vast majority of these parasites also harbor a non-photosynthetic plastid of secondary endosymbiotic origin referred to as the apicoplast, which houses critical metabolic functions needed for parasite survival. These parasites cause different types of infectious diseases in a wide range of animals and humans, including malaria, toxoplasmosis and babesiosis to name a few. Together, these diseases cause enormous economic and health burden worldwide. Drug resistance, toxicity issues and lack of effective second line of drugs, are the major issues affecting the clinical outcome of these infections. Concerted efforts from academic as well as public private partnership programs have identified hundreds of potent anti-parasitic molecules (example, the World Health Organization and Medicines for Malaria Venture coordinated “Malaria Box” and “Pathogen Box” compound collections).However, studies focused on mechanism of action and target identification for these “hit” molecules are necessary for generating “leads” that can progress through the drug discovery pipeline to become future medicines.

Many of these molecules have the potential to become next generation anti-parasitic drugs, but converting these “hits” molecules to “lead” candidates for drug discovery requires focused medicinal chemistry, biological mechanism studies (including target identification and *in vivo* efficacy studies), and pharmacological studies. While the entire drug discovery process is a challenging one, a major bottleneck in this process involves unraveling the mechanism of action for novel inhibitors. The overall focus of my thesis was to identify the mechanism of action for

novel and potent inhibitors of *Toxoplasma gondii*, an apicomplexan parasite, which causes toxoplasmosis in humans, and which can infect virtually any warm blood animal. The initial part of this thesis work involves the identification of novel small molecule inhibitors of *T.gondi i*growth using whole cell based screening assays. Next, we have focused on a set of inhibitors which cause delayed death of *T. gondii* by interfering with plastid segregation during parasite cell division. In the final part of this thesis work, we have carried out detailed mechanistic and target identification studies and for selected molecules affecting apicoplast biology. A brief description of the major findings and outcome of this thesis is detailed below.

Identifying molecules with anti-toxoplasma bioactivity using whole cell assays

Whole cell assays were used to identify inhibitors of parasite growth *in vitro*. A transgenic parasite line expressing the firefly luciferase gene was used in these screens, and the luminescence readout was used to quantify parasite growth inhibition. Overview of screening strategy is represented in ***Figure 5.1***.

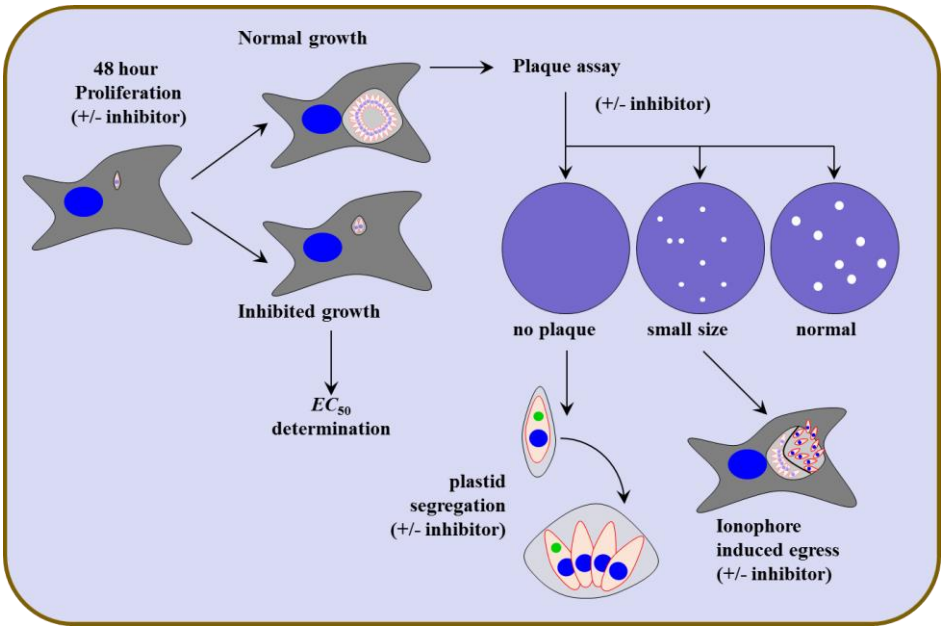

Figure 5.1: **Overview of the phenotypic assays used for identifying mechanistically distinct novel inhibitors of *T. gondii* growth.**

Three different chemical libraries (MMV Malaria Box [390 molecules], MMV Pathogen Box [400 molecules], and Sigma LOPAC, [1280 molecules]) were screened against tachyzoite stage *T. gondii*. To summarize the results from the above work, a total of>2000 molecules from the three libraries were screened, of which 338 molecules were found to inhibit parasite growth by ≥80% at 10 μM concentration. The potency of these molecules varied, and 122 molecules with nanomolar potency (EC_{50}<1μM) were identified. Out of these 122 molecules, 41 were found to be nanomolar inhibitors of *Plasmodium falciparum* growth as well. Thus, it is likely that these 41 molecules might be acting on conserved targets (and hence by similar mechanisms) in these two parasites. More than 700 molecules, which affected parasite growth by ≤20%, were screened by plaque forming assays to identify 73 delayed death inhibitors and 10 egress inhibitors. After identifying these molecules, we focused on further phenotypic and mechanistic studies for selected delayed death inhibitors of *T. gondii*.

Apicoplast phenotype studies on delayed death inhibitors

Apicomplexan parasites harbor a relict non-photosynthetic plastid organelle, termed apicoplast. This organelle houses essential metabolic pathways, such as Type II fatty acid biosynthesis, isoprenoid biosynthesis by the non-mevalonate pathway and iron-sulfur (Fe-S) cluster biosynthesis. This organelle also harbors its own circular genome, which is replicated and transcribed using prokaryotic pathways. Prior studies have shown that antibiotic inhibitors of bacterial protein synthesis (such as chloramphenicol) were able to kill *T. gondii*. Interestingly, these inhibitors did not kill the parasite immediately, but did so after a delay of one round of vacuolar replication. Hence, the term “delayed death” was used to describe the delayed growth

inhibition effect. Further studies have shown that delayed death of parasite resulting from inhibition of apicoplast housekeeping functions (such as protein synthesis) affects the segregation of the apicoplast to daughter cells during cell division, resulting in the formation of plastid-less daughter cells. This characteristic apicoplast phenotype was used to screen the 68 delayed inhibitors, which we had earlier identifyed from our screening studies. At least 29 of these inhibitor molecules were found to induce plastid missegregation in *T. gondii*.

It is probable that the molecules which are inducing apicoplast missegregation in *T. gondii* are targeting and inhibiting proteins with essential housekeeping functions in the apicoplast. If this is the case, and if the target for one or more of the delayed deathinhibitor is conserved in the phylogenetically related parasite *P. falciparum*, then those inhibitors will possibly affect the apicoplast of the malaria parasite and cause its delayed death as well. Therefore, we have tested all inhibitors affecting *T. gondii* apicoplast segregation on *P. falciparum* as well. The inhibitors found to cause delayed death in *P. falciparum* were validated using the isopentenyl pyrophosphate (IPP) rescue assay. These experiments resulted in the identification of inhibitors affecting the plastid and causing delayed death in both parasites. Further, for some of these inhibitors, we have carried out mechanistic and target identification studies as described in the next section.

Mechanism of Action (MOA) and target identification studies for selected delayed death inhibitors

In order to identify the target and study the MOA for selected inhibitors, we have used a genetic approach, wherein mutants, which are resistant to the inhibitor of interest are generated by chemical mutagenesis and then the mutation(s) responsible for the resistant phenotype are mapped. As per previous reports with drugs like atovaquone, resistance appears when the target

gene is mutated. However, it is also possible that resistance mechanism can be independent of MOA, as is the case with chloroquine resistance in *P. falciparum*. In this study, we have used N-ethyl-N-nitroso urea (ENU) mediated chemical mutagenesis to generate mutant populations, which become resistant to delayed death inhibitors. Resistance was verified by plaque forming assays, in which the mutants were able to proliferate and create plaques in the presence of drug at concentrations that would normally kill wild type parasites. Among the molecules tested, robust resistance was achieved for two molecules - MMV688471 (EC_{90} 5 µM) and MMV688345 (EC_{90} 2.5 µM) - for which the resistant parasites continued to proliferate in the presence of 20 µM and 10 µM of molecules respectively.

Clonal isolates of these mutants were obtained and their genomes were sequenced using the Illumina next generation sequencing platform. The mutant genomes (from three clonal lines for each molecule) were aligned to the parental wild type genome, and all SNPs and indels, which resulted in missense mutations, were identified. After further filtration using criteria such as expression in tachyzoite stage and essentiality, a few genes were narrowed down as probable candidates conferring resistance to the inhibitor in question. These candidate genes are being validated for their ability to confer resistance by gene replacement studies.

Overall summary of thesis

This thesis compiles chemical-genetic phenotyping studies in two apicomplexan parasites - *T.gondii* and *P. falciparum*, which resulted in the identification of delayed death inhibitors of these parasites. For selected delayed death inhibitors we have successfully addressed the mechanism of action. Overall this thesis work provides comprehensive documentation of selective phenotypic effects of MMV molecules against *P. falciparum* and *T. gondii*. By employing complementary techniques, we have prioritized more than 30 inhibitors that

selectively impair apicoplast segregation and parasite egress in both of these parasites. Few of these hits are “drug-like” molecules with proven antimalarial efficacy and novel chemical characteristics that make them amenable to subsequent medicinal chemistry work. Further investigations focused on dissecting the mode of action for these molecules using biochemical and genetic approaches would be helpful to identify novel targets for antiparasitic drug development. Ultimately, it is important to have novel therapeutic options against malaria and toxoplasmosis, which are two important human infectious diseases.

Appendix I

LOPAC molecules with nano molar potency in *T. gondii* (Page no-44)

Name	**Class**	**Mechanism of action**	***EC*$_{50}$ (µM)**
PD-180970	Cell signaling/ tyrosine kinase	PD-180970 is a potent inhibitor of the p210 Bcr-Abl tyrosine kinase.	0.05
Brefeldin A	Cytoskeleton and ECM	Fungal metabolite that disrupts the structure and function of the Golgi apparatus	0.11
CP-100356 monohydrochloride	Cell Signaling	CP-100356 is a specific inhibitor of MDR1 (P-Gp), the protypical ABC transporter. CP-100356 also inhibits prazosin transport in human breast cancer resistance protein (BCRP)-transfected MDCKII cells	0.06
CGP-74514A hydrochloride	Phosphorylation	Cdk1 inhibitor	0.98
Artemether	Immunomodulators and Antib	Artemether is a methyl ether derivative of artemisinin. It is used against multi-drug resistant strains of falciparum malaria.	0.31
Calcimycin	Intracellular Calcium	Ca2+ ionophore used to potentiate responses to NMDA, but not quisqualate glutamate receptors	0.19
Dequalinium chloride hydrate	K+ Channel	Selective blocker of apamin-sensitive K+ channels	0.39
Diphenyleneiodonium chloride	Nitric Oxide	Endothelial nitric oxide synthase inhibitor	0.06
Emetine dihydrochloride hydrate	Apoptosis	Apoptosis inducer; RNA-Protein translation inhibitor	0.38
5-Fluorouracil	Cell Cycle	Thymidylate synthetase inhibitor; leads to accumulation of cells in S phase	0.59
5-fluoro-5'-deoxyuridine	DNA Metabolism	DNA synthesis inhibitor; antiproliferative	1.00
Ivermectin	Cholinergic	Positive allosteric modulator of alpha7 neuronal nicotinic acetylcholine receptor; also modulates glutamate-GABA-activated chloride channels	0.25
PD-161570	Tyrosine kinase	PD-161570 is an inhibitor of human FGF-1 receptor tyrosine kinase.	0.50

L-703,606 oxalate salt hydrate	Tachykinin	Potent and selective non-peptide NK-1 tachykinin receptor antagonist	0.57
BIO	Phosphorylation	Potent, selective, reversible, and ATP-competitive glycogen synthase kinase 3alpha/beta (GSK-3alpha/beta) inhibitor.	0.97
MRS 1523	Adenosine	Selective A3 adenosine receptor antagonist in rat	0.76
Gossypol	Apoptosis	Natural product from cotton seeds with a variety of cell biological activities. Proapoptotic, antimalarial, PKC inhibition.	0.88
T0070907	Gene Regulation	T0070907 is very similar in structure and activity to the PPARgamma antagonist GW9662. T0070907 is more potent and has higher selectivity for PPAR-gamma over all other subtypes (about 800-fold)	0.89
Niclosamide	Antibiotic	Protonophoric anthelmintic; uncouples oxidative phosphorylation	0.12
PD173952	Kinase	PD173952 is a Src family kinase inhibitor.	0.64
Auranofin	Phosphrylation	Inhibits the release of inflammatory mediators from human macrophages, basophils, and pulmonary mast cells. Inhibits 5-lipoxygenase in human neutrophils. Is a potent inhibitor of selenoenzyme thioredoxin reductase (TrxR). Auranofin inhibits IKB kin...	0.57
Rottlerin	Phosphorylation	PKC and CaM kinase III inhibitor	0.47
Carmofur	Apoptosis	Carmofur is a derivative of fluorouracil, an antimetabolite used as an antineoplastic agent.	0.54
BAY 61-3606 hydrochloride hydrate	Phosphorylation	Spleen tyrosine kinase (Syk) inhibitor; anti-inflammatory	0.45
Tyrphostin AG 879	Phosphorylation	Tyrosine kinase nerve growth factor receptor (TrkA) inhibitor; inhibits 140 trk protooncogene and HER-2	0.61
U-74389G maleate	Cell Stress	Free radical lipid peroxidation inhibitor	0.95
Tyrphostin A9	Phosphorylation	Selective PDGF tyrosine kinase receptor inhibitor	0.11

Appendix II

MMV molecules having nano molar potency in both *T. gondii* and *P. falciparum* (Page no-48)

MMV ID	***T. gondii*** EC_{50} **(µM)**	***P. faciparum*** EC_{50} **(µM)**
MMV010576	0.71	0.19
MMV019189	0.14	0.91
MMV021013	0.28	0.72
MMV023227	0.52	0.81
MMV024397	0.20	0.56
MMV671636	0.01	0.01
MMV675968	0.53	0.07
MMV676477	0.06	0.76
MMV688754	0.05	0.01
MMV689480	0.01	0.01
MMV000304	0.88	0.53
MMV000326	0.47	0.89
MMV000720	0.79	0.89
MMV000963	0.76	0.10
MMV006169	0.71	0.18
MMV006309	0.26	0.52
MMV006319	0.88	0.15
MMV006389	0.92	0.28
MMV006513	0.17	0.20
MMV006825	0.65	0.12
MMV007127	0.80	0.59
MMV007875	0.56	0.48
MMV007977	0.03	1.00
MMV019918	0.21	0.76
MMV084434	0.01	0.88
MMV396669	0.10	0.14
MMV645672	0.21	0.25
MMV665826	0.91	0.23
MMV665886	0.26	0.46
MMV665940	0.02	0.66
MMV665941	0.09	0.05
MMV665977	0.91	0.21
MMV666596	0.78	0.87
MMV666687	0.84	0.22

Appendix III

MMV molecules which inhibits parasite growth in delayed manner and inhibits plaque formation for *T. gondii* (Page no-48)

Serial No	MMV ID	Serial No	MMV ID
1	MMV396693	35	MMV676571
2	MMV666067	36	MMV688471
3	MMV000848	37	MMV676476
4	MMV666109	38	MMV019721
5	MMV007906	39	MMV676386
6	MMV008455	40	MMV000011
7	MMV001049	41	MMV020152
8	MMV665857	42	MMV020321
9	MMV396633	43	MMV000858
10	MMV396717	44	MMV687803
11	MMV007092	45	MMV676204
12	MMV001041	46	MMV688327
13	MMV020885	47	MMV688466
14	MMV020439	48	MMV688846
15	MMV001038	49	MMV689028
16	MMV006820	50	MMV688508
17	MMV665969	51	MMV688361
18	MMV396672	52	MMV689029
19	MMV009127	53	MMV687248
20	MMV666125	54	MMV688124
21	MMV006937	55	MMV687146
22	MMV008212	56	MMV687170
23	MMV665891	57	MMV676053
24	MMV665946	58	MMV688844
25	MMV665785	59	MMV688178
26	MMV019199	60	MMV687798
27	MMV019110	61	MMV007920
28	MMV007113	62	MMV688345
29	MMV008160	63	MMV030734
30	MMV007160	64	MMV687729
31	MMV688416	65	MMV687813
32	MMV687762	66	MMV676270
33	MMV688942	67	MMV688990
34	MMV688756		

Annexure IV

Primers used this study

Primer Name	**Primer sequence(5'-3')**
Luc F'	GATCAGATCTATGGAAGACGCCAAAAACAT
Luc R'	GCATGCTAGCCACGGCGATCTTTCCGCCCTTCTTGGC
Tpi tag F'1	GATCGTAAGCTTAGTCTTGAGTGAACAGCTTGAGGTAC
Tpi tag R'1	GTCGTACCTAGGGGCTTGCTGCTTCGCTGCATCAATG
Tpi tag conf F'2	CGAAGAAAGTGCGCGCGGCGCTCAAC
Tpi tag conf R'2	CCATGATATAGACGTTGTGGCTGTTGTAG
Tpi tag conf R'3	TCTAGAACTAGTGGATCCCCCTCCACC