APICOPLAST ISOPRENOID PRECURSOR SYNTHESIS AND MOLECULAR BASIS OF FOSMIDOMYCIN RESISTANCE IN TOXOPLASMA GONDII

by

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(Under the Direction of BORIS STRIEPEN)

ABSTRACT

Apicomplexan parasites are important pathogens responsible for economically important as well as life threatening infections in humans and animals. Poor efficacy and emerging resistance against available drugs is a matter of concern, as drug treatment still remains the chief way to combat these infections. The identification of a divergent plant plastid like organelle called apicoplast in these parasites has invited much attention for the development of new drugs. This unique organelle is essential as it is used by these parasites to meet its metabolic demands.

Apicoplast supplies three important metabolites, fatty acids, isoprenoid subunits and heme. Out of these three metabolites, pathway to make isoprenoid subunits seems to be the most important one evidenced by its conservation among all the members of the phylum. The pathway is also the target of the antibiotic fosmidomycin, a drug that is in the phase II of clinical trials to be used against *Plasmodium*, the causative agent of malaria. Although effective against *Plasmodium* and *Babesia*, many other members of

the Phylum including *Toxoplasma gondii* is naturally resistant to the antibiotic at concentrations 100 fold higher than against the sensitive parasites. I have used genetic tools to dissect this surprising observation using *Toxoplasma* as the model organism. Using the conditional knock out strategy in *Toxoplasma*, we showed that the target pathway is essential in organisms resistant to fosmidomycin. By complementing the target enzyme with a known fosmidomycin sensitive enzyme, we then realized that lack of drug access is the reason for resistance. In support of this hypothesis, we engineer denovo drug sensitive *T.gondii* parasites by the heterologous expression of the bacterial glycerol-3-po4 transporter capable of importing fosmidomycin. This sensitivity engineered by the transporter expression in *Toxoplasma* was comparable to what is seen in *Plasmodium* and *Babesia*. Further biochemical analyses showed that parasite plasma membrane is the chief barrier for drug entry as the transporter-expressing parasites accumulated the drug in the cytoplasm with the help of the transporter. Lethal infections with these parasites in mice could be treated with intraperitoneal injections of fosmidomycin validating the chemotherapeutic potential of the pathway.
INDEX WORDS: Apicoplast, *Toxoplasma*, Fosmidomycin, Apicomplexa

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

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DEDICATION

TO MY HIGH SCHOOL TEACHERS, yes I've come this far.

ACKNOWLEDGEMENTS

Thank you, Boris Striepen for the most memorable parties, outings, for the bike, for two international conferences, for stimulating discussions, for pushing me to the limits and most importantly for helping me to know myself.

Thank you, Slivia Moreno for the helpful discussions and suggestions, Rick Tarleton and Dan Colley for their creative inputs and Roberto Docampo for his willingness to help me submit the thesis in a timely manner.

Thank you, Carrie Brooks for helping me with my thesis project, making “labing” less stressful by making each of us feel special.

Thank you, people in McFadden lab (Univ of Melbourne, Australia) and Song lab (Bayor college, TX) for their contributions to my project

Thank you, Lisa Sharling for stepping-stones, Carly Jordan for being an honest good friend and all the present and past members of the Striepen lab for their encouragement and help.

Thank you, Family for being patient, my wife Leena for helping me cope with the turbulences associated with graduate studies and to my Mom for laying the foundations of excitement associated with knowing things.

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CHAPTER 1

INTRODUCTION

The phylum Apicomplexa is a group of parasitic protists responsible for economically important human and animal diseases. *Plasmodium*, the agent of malaria is the most important member. *Toxoplasma* and *Cryptosporidium* are parasites responsible for diseases in immunocompromised individuals. *Toxoplasma* infection is usually asymptomatic, however severe disease is clinically evident in immunocompromised individuals (Jones et al., 2006) The members of the phylum are also responsible for economically important animal diseases such as babesiosis, coccidiosis and theileriosis. Lack of an effective preventive vaccine or a therapeutic combination with minimal side effects against most of these pathogens is an important concern (Petersen, 2007). Drug treatment still forms the major way to combat these infections and there is a constant need to identify new targets for therapeutic interventions in the parasite.

The discovery of a plastid like organelle called apicoplast in these parasites shed fresh light into the drug discovery process. This organelle is not present in the mammalian hosts and therefore may be a good target for treatment. Although it was long noticed in electron micrographic studies (Siddall, 1992), the function and origin of apicoplast remained enigmatic for many years. Molecular biological studies aimed at

identifying the mitochondrial genome of the malaria parasite discovered an unusual 35 kb DNA element that has features reminiscent of a chloroplast genome: inverted subunits of ribosomal RNA and subunits of a prokaryote type RNA polymerase (Gardner et al., 1991a; Gardner et al., 1991b; Gleeson, 2000; McFadden and Waller, 1997; Wilson et al., 1996). Key experiments using in situ hybridization coupled with transmission electron microscopy localized this genome to the multi membrane organelle of *T. gondii* (Kohler et al., 1997; McFadden et al., 1996). This organelle was called as apicoplast based on its presence in the phylum Apicomplexa and its phylogenetic relationship to plastids. This surprising discovery of the apicoplast in parasites led to important questions: What is the origin of the apicoplast and what are the major functions of the apicoplast in the parasite? Endosymbiosis of a free living photosynthetic eukaryote by another eukaryote is now widely accepted as the evolutionary mechanism that led to this organelle in apicomplexan parasites (Marechal and Cesbron-Delauw, 2001). With time, most of the genes of the endsymbiont were transferred to the host nucleus. Furthermore its structure and functions were simplified until essentially only a plastid remained with its small genome. However, this plastid remained bounded by four membranes. In addition to the two chloroplast membranes there are two extra membranes derived from the algal plasma membrane and the presumptive phagosomal vacuole of the host. These extra membranes are a tell tale of this organelle's evolution through secondary endosymbiosis (Kohler et al., 1997; Waller and McFadden, 2005).

The size of the apicoplast genome in *Toxoplasma gondii* is only 35 kb, and it primarily encodes genes for functions related to maintenance of the organellar genome itself, that is, transcription and translation (Wilson et al., 1996). The initial benefit of the acquisition of a plastid by the apicomplexan ancestor might have been the photosynthetic capabilities associated with the organelle. However, many of the lineages derived for this endosymbiosis, including the apicomplexan parasites, retained the organelle, most likely due of the metabolic benefits associated with its presence. The metabolic functions associated with the apicoplast are mediated by nucleus encoded apicoplast targeted [NEAT] proteins. These proteins are targeted to the apicoplast by means of a bipartite leader sequence at the N-terminus (Foth et al., 2003). This leader sequence consists of a classical secretory signal peptide, which directs the co translational insertion of the protein to the endomembrane system of the parasite. Downstream of the signal peptide there is a so-called transit peptide which directs the protein to the apicoplast (Waller et al., 2000). These peptides are variable in length, from 25 to over 100 amino acids, and show little conservation at the primary sequence level (Bruce, 2001). Three important anabolic pathways have been identified in the apicoplast 1) to synthesize fatty acids 2) isoprenoid subunits and 3) heme precursors. The heme synthesis pathway is shared between the apicoplast and the mitochondrion of the parasite while the other two pathways are exclusive for the apicoplast. *Plasmodium*, *Toxoplasma* and *Eimeria* maintain all three pathways, while *Babesia* and *Theileria* only have the isoprenoid pathway (Ralph et al., 2004). All these pathways are excellent candidates as targets to develop drugs against the apicomplexan parasites, as

they are divergent from their host cell counterparts. With the identification of these pathways, questions arise concerning the importance of these pathways for the survival of these organisms. The parasites' requirement for the products of these pathways in turn likely reflects the requirement for the apicoplast in more general terms. Answering this question may also address the reason behind the retention of this organelle despite the loss of its initial photosynthetic benefits. The fatty acid pathway has been studied extensively in *Plasmodium* and *Toxoplasma* using genetic and pharamacological tools. The pathway is essential in *Toxoplasma*, but its functions beyond the apicoplast are still unclear (Mazumdar et al., 2006). *Plasmodium* needs the apicoplast fatty acid pathway only for the late liver stages and can dispense it during the early liver stages, blood stages and the mosquito stages (Vaughan et al., 2009; Yu et al., 2008). Both *Plasmodium* and *Toxoplasma* have additional systems to synthesize fatty acids potentially making the apicoplast pathway redundant (Mazumdar and Striepen, 2007). The heme pathway has not been studied in as much detail as the FASII pathway, but some of the enzymes involved have been characterized in *Toxoplasma* and *Plasmodium* (Nagaraj et al., 2009a; Nagaraj et al., 2008; Nagaraj et al., 2009b; Shanmugam et al.). There are also claims that the parasite may be able to salvage enzymes involved in the pathway from the infected host cell (Bonday et al., 2000; Varadharajan et al., 2004). Which, if any parts of the heme pathway are truly essential remains to be established. The isoprenoid precursor pathway on the other hand is the most conserved plastid pathway among apicomplexans and is the target of the drug fosmidomycin. This drug shows excellent activity against *Plasmodium* and *Babesia* and

is in clinical trials to be used as an antimalarial. But surprisingly, the drug has poor activity against many other members of the phylum. Using *Toxoplasma* as a model, I have addressed this conundrum. The important questions that I have asked are: 1) Is the DOXP pathway essential in organisms resistant to fosmidomycin? And if so what is the mechanism of resistance, 2) Are parts of the pathway made redundant by other metabolic pathways? 3) Is the target inherently resistant to the drug? 4) Is the drug not reaching its target?

This dissertation is divided into four chapters. Chapter 2 is a review of the literature to detail the biochemical reactions, therapeutic targets in the pathway, reasons that govern sensitivity to available drugs, and the role of the pathway in parasite metabolism. Chapter 3 describes my work answering the questions outline above by experimentally testing the essentiality of the pathway and the potential reasons for its resistance to the antibiotic fosmidomycin. The conclusions that I have drawn from my work are discussed in Chapter 4 along with future directions aimed to unravel the metabolic roles of the apicoplast isoprenoid pathway.

CHAPTER 2

REVIEW OF LITERATURE

ISOPRENOID NEEDS OF APICOMPLEXA: UNDERSTANDING THE ROLES OF APICOPLAST LOCALIZED PATHWAY TO MAKE ISOPRENOID PRECURSORS

2.1. INTRODUCTION

The phylum Apicomplexa is comprised entirely of parasitic protists, including many pathogens of medical and veterinary importance. The most prominent member is *Plasmodium*, the agent of malaria. The members are also responsible for causing economically important animal diseases such as babesiosis, coccidiosis and theileriosis. *Toxoplasma* and *Cryptosporidium* cause disease in immunocompromised individuals. About 30% of world's population is infected with *Toxoplasma*, most of whom experience no overt disease symptoms. *Toxoplasma* is also capable of travelling transplacentally in infected pregnant women with resultant morbidity and even mortality of the child. Lack of effective preventive vaccines and emerging drug resistance against infections caused by apicomplexan are a pressing issue. Surprisingly, most apicomplexan parasites possess a non-photosynthetic plastid. The organelle, now known as the apicoplast, is clearly visible in electron micrographs with its distinctive four surrounding membranes (McFadden et al., 1996); (Ferguson et al., 2005). While there is

consensus over the fact that the plastid in apicomplexan parasites came through an evolutionary endosymbiotic event, there is some difference of opinion regarding the source of the plastid (Gleeson, 2000), some authors favor red some green algal lineage ancestry. (Keeling, 2009; Kohler et al., 1997; McFadden et al., 1996; Wilson et al., 1996). The evidence supporting red ancestry appears to accumulate steadily making this the most likely scenario at this time (Keeling, 2009). All members of the phylum except *Cryptosporidium* harbor an apicoplast (Zhu et al., 2000). Bereft of the immediate and obvious benefit of photosynthesis in the initial phase of the endosymbiotic relations, the organelle survived evolution, most likely because it is the home for vital metabolic pathways required essential to the parasite (He et al., 2001; Ralph et al., 2004). So far, three important pathways have been identified in the apicoplast 1)a fatty acid pathway, 2)an isoprenoid precursor pathway 3) and a heme biosynthesis pathway (Ralph et al., 2004). While the heme synthesis pathway is shared between mitochondrion and apicoplast, fatty acid and isoprenoid precures pathways are not shared with any other organelle. The extent of the gene transfer between endosymbiont and host organism is evident by the fact that all the enzymes that constitute these pathways are nuclear encoded and posttranslationally targeted to the apicoplast using a bipartite signal. Many of these metabolic pathways have been studied in detail with therapeutic intentions. They are of (ultimately) prokaryotic origin and different of the pathways used by the mammalian host. Promising results have been obtained using pharmacological approaches (Wiesner and Jomaa, 2007; Wiesner et al., 2008; Wiesner and Seeber, 2005). These potential new targets have to be further validated by additional

experiments to fully understand their value. An important and maybe the most important metabolic pathways hosted exclusively by the apicoplast provides the cell with the isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). This review aims to detail the biochemical reactions, therapeutic targets, mechanisms that govern susceptibility to drugs and the importance and role of the pathway in parasite metabolism.

2.2 APICOPLAST: ISOPRENOID PRECURSOR FACTORY OF ALL APICOMPLEXANS

Isoprenoids are a large family of molecules with important biological functions including cellular respiration, photosynthesis, hormone-based signaling, protein modification and they form important structural components of cellular membranes. Isoprenoids are made by sequential addition of five carbon compounds the universal precursors IPP and DMAPP. Two important anabolic pathways have been identified that lead to these precursor compounds: the mevalonate pathway used in animals and fungi and many other eukaryotes, and the non-mevalonate pathway used by most bacteria and the plastids of plants (Hunter, 2007). In congruence with its algal plastid ancestry, the apicoplast is home to the non-mevalonate pathway. This is the only pathway so far identified in this phylum to synthesize isoprenoid precursors. *Cryptosporidium*, which does not have an apicoplast, lacks isoprenoid precursor synthesis (Clastre et al., 2007; Zhu et al., 2000). The pathway has seven catalytic reactions, these are distinctly

different from the host mevalonate pathway, yet both pathways culminate in the production of IPP and DMAPP [Fig. 2.1].

All apicoplast biosynthetic pathways are likely fed through the import of intermediates of glycolysis from the parasite cytoplasm using the apicoplast phosphate transporter associated with the four membranes of the apicoplast. *Toxoplasma* has a single transporter likely associated with all four membranes of the apicoplast (Fleige et al., 2007; Karnataki et al., 2007). This transporter has been shown to be essential for the parasite and is the source of carbon and energy for the apicoplast (Brooks et al., 2010). *Plasmodium*, on the other hand, has two transporters that associate with different membranes of the apicoplast (Mullin et al., 2006); (Lim et al., 2010). The ability of these transporters to import phosphoenol pyruvate (PEP) as well as triose phosphates like dihydroxy acetone phosphate (DHAP) has also been documented biochemically for recombinant protein (Brooks et al. 2010; Lim et al., 2010). The imported PEP and DHAP can act as substrates for the synthesis of pyruvate and glyceraldehyde-3-po4 (G3P) in the lumen of the apicoplast (Fleige et al., 2007; Ralph et al., 2004). Pyruvate and G3P are the initial metabolites for the non-mevalonate isoprenoid precursor pathway that are condensed to yield 1-deoxy-D-xylulose-5 phosphate (DOXP, I will refer to the non-mevalonate pathway as DOXP pathway from here on). DOXP synthase catalyses this first step of the DOXP pathway. This is considered the rate limiting step of the entire pathway in plants as DOXP levels correlate with levels of isoprenoids (Eisenreich et al., 2004). DOXP synthase expression also acts as the point of transcriptional regulation for the isoprenoid pathway in

Mycobacterium tuberculosis. Overexpression of this enzyme in *Mycobacterium* resulted in increased flux of metabolites through the pathway and increased the expression levels of the downstream enzymes in the pathway (Brown et al., 2010).

The next step of the pathway is catalyzed by the enzyme 1-deoxy-D-xylulose-5 phosphate reductoisomerase (DOXPRI) [Fig.2.1]. This enzymatic reaction involves a skeletal rearrangement of DOXP to yield the intermediate 2C-methyl-D-erythrose-4 phosphate, which is then reduced using NADPH to yield 2C-methyl-D-erythritol-4-phosphate (MEP). The kinetic as well as structural properties of the *E. coli* homologue of this enzyme have been studied extensively. The recombinant enzyme catalyzed the formation of MEP from DOXP in a single catalytic step in the presence of NADPH and required a divalent cation such as Mn^{2+}, Mg^{2+} or Co^{2+}. The enzyme forms a physiologically active homodimer and has three domains, an N-terminal NADPH binding domain, a connective linker domain and a C-terminal domain. The active site of the enzyme where the substrate DOXP binds spans the connective and the C-terminal domain (Kuzuyama et al., 2000; Mac Sweeney et al., 2005; Takahashi et al., 1998). Recently, a new family of enzymes catalyzing this committing step and able to complement *E.coli* DOXPRI mutants was identified in *Brucella abortus* (Sangari et al., 2011).

MEP is converted to a cyclic diphosphate form by the sequential action of three enzymes (Hunter, 2007) [Fig.2.1]. The initial transfer of a nucleoside phosphate moiety (usually cytosine dinucleotide phosphate) is catalyzed by the enzyme 4-diphosphocytidyl-2C-methyl-D-erythritol-4-phosphate synthase (IspD). This is followed

by the phosphorylation of the hydroxyl group in the original methyl-D-erythritol backbone by a kinase utilizing ATP (IspE). This phosphorylated intermediate is then converted to its cyclic diphosphate by the release of the nucleoside monophosphate (IspF). In the next step of the pathway, the cyclic diphosphate is reduced with the opening of the ring structure to yield 1-hydroxy-2-methyl-butenyl 4-phosphate (HMBPP) by the enzyme HMBPP synthase (HMBPS). HMBPP is further reduced by LytB to yield the final products of the pathway: IPP and its allyl-isomer DMAPP (Eisenreich et al., 2004) [Fig.2.1]. Many organisms have an isomerase enzyme which catalyses the interconversion of IPP and DMAPP, but none has been identified in apicomplexan parasites (Lee and Poulter, 2006). This suggests that LytB is the final enzyme of the pathway and probably mediates the synthesis of both IPP and DMAPP. Such a capability of LytB to generate both IPP and DMAPP was also observed in *E. coli* cells overexpressing recombinant LytB (Rohdich et al., 2002). The final two reductases, HMBPP synthase and LytB, are enzymes containing Fe-S clusters in its core (Beinert and Kiley, 1999; Hunter, 2007).

IPP and DMAPP are five carbon compounds that act as the precursors of isoprenoid chains. Polyisoprenoids are synthesized by the sequential addition of these basic building blocks. Two isoprenoid precursors can yield geranyl pyrophosphate (GPP), three result in farnesyl pyrophosphate (FPP), and four in geranyl geranyl pyrophosphate (GGPP). Farnesyl pyrophosphate synthase catalyzes the synthesis of GPP and FPP, while GGPP synthase makes GGPP. *Toxoplasma* has a bifunctional FPP synthase that mediates both these enzymatic reactions (Ling et al., 2007). A

functional geranyl geranyl pyrophosphate synthase has also been characterized in *Plasmodium* recently. This enzyme can use DMAPP, FPP or GPP as the substrate to generate GGPP (Artz et al., 2010). There is also some evidence showing the ability of apicomplexan parasites to salvage isoprenoids from their hosts (Chakrabarti et al., 1998; Chakrabarti et al., 2002; Ibrahim et al., 2001).

2.3 FOSMIDOMYCIN RESISTANCE IN APICOMPLEXA: THOUGHTS AND REASONS.

2.3.1 Fosmidomycin, the drug that targets apicoplast isoprenoid precursor biosynthesis

The apicoplast localized DOXP pathway for isoprenoid biosynthesis is viewed as a promising drug target (Wiesner and Jomaa, 2007; Wiesner et al., 2008; Wiesner and Seeber, 2005). While the enzymes involved in the DOXP pathway in apicoplast are unique to the parasite, the downstream enzymes that utilize these precursors have homologues in the mammalian host cell. The most prominent antibiotic that targets this pathway is fosmidomycin, a structural analogue of DOXP, the first intermediate of the pathway (Jomaa et al., 1999; Zhang et al., 2011). Fosmidomycin binds to DOXP pocket of the catalytic site of DOXP reductoisomerse thereby inhibiting the binding and subsequent catalytic conversion of DOXP to MEP (Mac Sweeney et al., 2005; Merckle et al., 2005; Proteau, 2004; Yajima et al., 2007). Fosmidomycin has also been shown to inhibit a new family of enzymes known to exhibit DOXP reductoisomerase like activity in *B. abortus* (Sangari et al., 2011). Fosmidomycin is active against *Plasmodium* (IC_{50}:0. 4-

1.2 μM) and *Babesia* (IC_{50}: 3-4 μM) (Jomaa et al., 1999; Sivakumar, 2008) and is currently in phase II of clinical trials to be used in combination with clindamycin (Borrmann et al., 2004a; Borrmann et al., 2005; Borrmann et al., 2004b; Borrmann et al., 2006; Jomaa et al., 1999; Olliaro and Wells, 2009). Surprisingly, the drug shows no activity against many other members of the phylum like *Eimeria*, *Toxoplasma* and *Theileria* at concentrations as high as 100 μM (Clastre et al., 2007; Lizundia et al., 2009). Researchers have also used derivatives of fosmidomycin. One such derivative is FR-900098, a methyl derivative of fosmidomycin that shows improved activity against *Plasmodium* when compared to fosmidomycin (Jomaa et al., 1999). The drug as ineffective against *Toxoplasma* as fosmidomycin (Fig. 2.2).

One important concern in understanding resistance is the validation of the drug target to rule out off target effects. Recently LC-MS based approaches have been used to validate the target of fosmidomycin in *P. falciparum* and *E. coli*. The study showed that fosmidomycin targets the apicoplast isoprenoid pathway, directly inhibiting DOXP reductoisomerase. The study also demonstrated that the drug also has an inhibitory effect on a second enzyme catalyzing the immediate downstream step of DOXPRI, methylerythritol phosphate cytidyl transferase [IspD] (Zhang et al., 2011). This conclusion was based on the observation that fosmidomycin treatment did not change the levels of MEP, the product of reaction catalyzed by the known target DOXP reductoisomerase. Instead, there was a consistent reduction of all the intermediates downstream of the catalytic step mediated by IspD. This observation was consistent in fosmidomycin treated cells of *P. falciparum* and *E. coli*. Additional evidence was

obtained by the ability to induce fosmidomycin resistance by overexpressing IspD in *E. coli* followed by the ability of fosmidomycin to inhibit purified IspD directly in an in vitro enzyme assay. This is the first direct biochemical validation of the target of fosmidomycin in an apicomplexan parasite (Zhang et al., 2011). This study hence supports the hypothesis that fosmidomycin is an inhibitor of the DOXP pathway in *Plasmodium*.

2.3.2: Barriers for fosmidomycin entry into the infected cell

Lack of drug access has been shown to be the reason for the resistance against fosmidomycin in *M. tuberculosis* and *B. abortus* (Brown and Parish, 2008; Sangari et al., 2011). Drug access could also be a potential reason for resistance in apicomplexan parasites. The barriers that fosmidomycin needs to cross in an infected parasitic cell are shown schematically in Fig.2.3. While the PVM is mostly permeable to small compounds, the host cell plasma membrane, the parasite plasma membrane and the apicoplast membranes are all potential impediments for fosmidomycin. The parasite species resistant to fosmidomycin infects nucleated cells. The susceptible species, *Plasmodium* and *Babesia*, have so far only been studied in the anucleated red blood cell stage. In a recent study it has been suggested that fosmidomycin uptake and sensitivity in *Plasmodium* and *Babesia* is dependent on new permeability pathways formed in the infected red blood cells (Baumeister et al., 2011). Drugs thought to inhibit the formation of new permeability pathways reduced the uptake of fosmidomycin in red blood cells infected with *Plasmodium* and *Babesia*. The antimalarial activity of

fosmidomycin in an in vivo mouse model was documented in the first report of the DOXP pathway in *Plasmodium* (Jomaa et al., 1999). Drug treatment reduced blood stage parasitemia, the specific effect of the drug on other stages of infection was not studied at that point. In an effort to evaluate the effect of intra-peritoneal fosmidomycin administration on liver stages of rodent malaria, Baumeister and colleagues quantified the liver load of infection by measuring the levels of parasite 18sRNA in the livers of infected mice (Baumeister et al., 2011). Primaquine, a drug that is known to target the liver stages was used as a positive control. The researchers did not detect an effect of fosmidomycin on the rRNA levels when compared to primaquine treatment and concluded that the drug is ineffective against liver stage infection. To understand the resistance in other members of the phylum, the authors also studied the enzymatic activity of DOXPRI in *Toxoplasma*, the target of fosmidomycin in parasite lysates. DOXPRI activity was observed in *Toxoplasma* lysates and this activity was sensitive to fosmidomycin in a dose dependent manner. The inability of *Toxoplasma* infected fibroblasts to take up radioactive FR900098, the methyl derivative of fosmidmycin, led to the authors to the conclusion that the host cells might play a major role in drug entry and sensitivity (Baumeister et al., 2011). Note that this study was conducted and published independently and in parallel to my thesis work. As will become clear in the following chapter while we agree with Baumeister in some aspects, there are important differences in the mechanism that we outline and I will discuss these in detail in the following chapters. The conclusions of the Baumeister study rest largely on negative observations as the lack of uptake and the lack of activity. While not without merit such

negative data have their inherent technical concerns that may limit the conclusions to be drawn. We feel that the strength of our arguments results from the fact that they are based on experimental settings that allow for gain of function observation. We support our model by genetically engineering a naturally resistant parasite into a fully fosmidomycin susceptible strain (Nair, et al., 2011).

2.4 DOXP PATHWAY AND ITS EXTENSIONS AS CHEMOTHERAPEUTIC TARGETS

2.4.1 Apicoplast DOXP pathway: Other known targets

One of the other drugs that targets the apicoplast DOXP pathway is ketoclomazone, a drug that inhibits the first enzyme of the pathway, DOXP synthase. Ketoclomazone is a derivative of clomazone, a herbicidal compound. The drug inhibited the growth of *E. coli* and *H. influenzae* and this inhibition could be reverted by the addition of DOXP to the media showing the specificity of the inhibitor (Matsue et al., 2010). This antibiotic also showed modest activity against *Toxoplasma* at higher concentrations in a standard fluorescent growth assay (Fig.2.2). The effect of ketoclomazone on other members of apicomplexa needs to be tested. Inhibition of LytB, the last enzyme of the pathway using ribozymes that selectively cleave LytB transcripts was also found to be lethal in *Plasmodium* validating the target enzyme (Vinayak and Sharma, 2007). Small molecules capable of inhibiting this enzyme have been developed and used as tools to interrogate LytB substrate binding and catalysis (Oldfield, 2010).

2.4.2 Potential drug targets among enzymes that utilize isoprenoid precursors

Considering the smaller economic potential associated with drug development against tropical diseases, an alternate strategy is to “piggy back” on available drugs that were initially developed for other purposed but affect parasite pathways as well. One such group of compounds are the bisphosphonates. Bisphosphonates are pyrophosphate analogues used to treat bone disease but that also show activity against a wide range of parasites. They target the enzymes utilizing the isoprenoid precursors synthesized in the apicoplast or salvaged from the host cell. The best characterized target of bisphophonate is the enzyme farnesyl pyrophosphate synthase which catalyzes the condensation of two isoprenoid precursors (Ling et al., 2007; Ling et al., 2005) [Fig.2.4]. Bisphosphonates have been found to be active against both *Toxoplasma* and *Plasmodium* in vitro and in vivo in studies in mice (Ghosh et al., 2004; Martin et al., 2001; Martin et al., 2002; Mukkamala et al., 2008; Rosso et al.; Shubar et al., 2008; Szajnman et al., 2001; Szajnman et al., 2003; Szajnman et al., 2005; Yardley et al., 2002). Bisphosphonates has also been shown to be active against *Cryptosporidium parvum* targeting a non-specific polyprenyl synthase enzyme that might be utilizing isoprenoids salvaged from the host (Artz et al., 2008). Bioavailability has been a problem in the development of potent bisphosphonate compounds and lipohilic bisphosphonates with better membrane permeability have been shown to be more potent against *Plasmodium* liver stages (Singh et al., 2010). Drugs that target sterol biosynthesis have also been shown to be effective against *Toxoplasma* (Dantas-Leite et al., 2004; Martins-Duarte et al., 2010; Martins-Duarte et al., 2006) [Fig.2.4]. The

only sterol identified so far in *Toxoplasma* is cholesterol and the ability of the parasite to salvage cholesterol from host cell resources is well documented (Coppens et al., 2000). This could mean that the parasite death is due to off target effects of the drugs or that the parasite has specialized sterol needs and yet unidentified pathways supplying these needs that are the targets of these drugs.

Most eukaryotes require protein prenylation for survival, a process by which geranyl or farnesyl residues are attached to proteins to aide in protein localization and/or function. The process and the enzymes involved in protein prenylation in parasites are discussed in detail in a separate section of this review below. However, protein prenyl transferase inhibitors have been shown to be effective against many parasites (Bendale et al., 2007; Eastman et al., 2006; Fletcher et al., 2008; Linares and Rodriguez, 2007).

2.5 FUNCTIONS OF THE DOXP PATHWAY [FIG.2.4]

2.5.1 DOXP: The thiamine source for apicomplexa?

There is some evidence suggesting the ability of apicomplexan parasites to synthesize metabolites that are commonly regarded as vitamins for humans (Gengenbacher et al., 2006; Wrenger et al., 2006; Wrenger et al., 2005). DOXP synthesized in the apicoplast could potentially serve as a precursor for the biosynthesis of both thiamine (vitamin B1) as well as pyridoxal phosphate (vitamin B6) (Sprenger et al., 1997). *Plasmodium* and *Toxoplasma* have candidate genes for the enzymes involved in thiamine biosynthesis (Muller and Kappes, 2007). The main function of

thiamine is to act as a cofactor for numerous enzymes involved in carbohydrate metabolism as well as the DOXP synthase enzyme itself (Ralph et al., 2004). The biosynthesis of thiamine involves the separate formation of hydroxy methyl pyramidine diphosphate (HMP-PP) and methyl thiazole phosphate (THZ-PP), THZ-PP is generated from DOXP. These two compounds are condensed to form thiamine monophosphate catalyzed by the enzyme thiamine monophosphate synthase (ThiE).The *Plasmodium* genome encodes a putative homologue of this enzyme (PFF0680c). Thiamine monophospahte is dephosphorylated before being phosphorylated again to generate thiamine pyrophosphate (Begley et al., 1999). The enzyme responsible for the final phosphorylation is thiamine phosphate kinase (TPK, PFI1195c) and has been identified in *Plasmodium* and interestingly the gene appears to encode an amino terminal extension resembling apicoplast leader (Gardner et al., 2002; Muller and Kappes, 2007; Ralph et al., 2004). *Toxoplasma* also encodes this kinase (33.m02662), but a signal peptide has not been identified so far (Muller and Kappes, 2007). Dephosphorylation of thiamine monophosphate is a prerequisite for TPK activity and a putative paranitrophenyl phosphatase potentially capable of this dephosphorylation step has was identified and characterized in *Plasmodium* (Knockel et al., 2008). Although *Plasmodium* appears to be equipped with the metabolic machinery for the thiamine biosynthesis, they need external supply of thiamine or at least the precursor 2-methyl 5-hydroxymethyl pyramidine (HMP). This is supported by the fact that continuous culture of *Plasmodium* in the absence of thiamine resulted in a growth defect that was overcome by external supply of thiamine or its precursor HMP (Divo et al., 1985;

Eschbach et al., 2006; Wrenger et al., 2006). The fact that they can survive in the presence of HMP, one of the precursors for the biosynthesis of thyamine may also suggests that they might be using DOXP as a source for the formation of the thiazole precursor, THZ-PP. Homologues of enzymes involved in the generation of THZ-PP from DOXP have been identified in *Plasmodium* and *Toxoplasma*. Salvage of the thiazole precursor is also a possibility (Muller and Kappes, 2007). Another potential use of the DOXP generated is to act as substrate for the biosynthesis of pyridoxal or vitamin B6 (Mittenhuber, 2001), but earlier analyses indicate that the parasites must be utilizing a DOXP independent pathway for the synthesis of B6. The presence of enzyme homologues for thiamine biosynthesis and the biochemical need of thiamine for parasite viability may suggest that the apicoplast isoprenoid pathway could provide the necessary precursor for this important metabolite.

2.5.2 Polyisoprenoids: Membrane anchors for proteins and sugar platforms

Polyisoprenoids are required for protein prenylation, the transfer of either farnesol or geranol to the carbox-terminal end of a target protein. The enzymes that catalyse this transfer are called prenyl transferases. Most of the prenylated proteins have a C-terminal CAAX, CXC or XCC recognition motif, where “C” stands for cysteine, “A” for any aliphatic amino acid and “X” for any amino acid. The prenyl group is linked to the cysteine residue via a thioester bond (Rilling et al., 1990). A diverse array of eukaryotic proteins are required to be prenylated to be functional, this includes numerous regulatory GTPases such as Ras, Rac, Rap, Rho and Rab (Takai et al., 1992), nuclear

lamins (Farnsworth et al., 1989), and a variety of protein kinases (Inglese et al., 1992). Some of these proteins, like Ras, are important mediators of cell cycle regulation. Prenylation of the target protein favors its membrane association.

Protein prenylation in apicomplexan parasites

Apicomplexan parasites like *P. falciparum and T. gondii* have been shown to prenylate proteins (Chakrabarti et al., 1998; Ibrahim et al., 2001). The first description of protein prenylation in *Plasmodium* identified both farnesyl transferase and geranyl geranyl transferase activity (Chakrabarti et al., 1998). *Plasmodium* red blood cell stages fed with radioactive farnesol and geranyl geraniol showed strong labeling of the intracellular protein pool suggesting uptake and likely prenylation. A prenylated protein pool could be observed at all stages of intracellular development in the red blood cell using a commercially available anti-farnesyl polyclonal antibody (Chakrabarti et al., 2002). These studies also suggested the ability of the blood stage parasites to take up FPP as well as GGPP and use them for protein modifications (detected here by radiloabelin). After labeling, radioactive Ras and Rab proteins could be immunoprecipitated from the cell using the antibodies specific for these proteins confirming prenylation. Anti Ras antibodies precipitated farnesyated protein while anti Rap antibodies precipitated geranyl geranylated proteins (Moura et al., 2001). Similarly a Rab1 homologue has been shown to be prenylated in the apicomplexan *Theileria parva* (Janoo et al., 1999). Recently, a potential protein tyrosine phosphatase with terminal prenylation motif has also been characterized in *Plasmodium*. Antibody staining

specific for the native enzyme shows that the enzyme localizes to the Golgi as well as to the micronemes suggesting a potential role in secretory trafficking and host cell invasion (Pendyala et al., 2008). In another recent study, a SNARE protein known to be involved in vesicle trafficking has been characterized in *Plasmodium*. A homologue of this protein called PfYkt6 in yeast is known to be involved in ER to Golgi trafficking and was found to be farnesylated and geranyl geranylated in *Plasmodium* (Ayong et al., 2011). Extracellular *T.gondii* tachyzoites have also been shown to take up radiolabelled farnesol and geranyl geraniol and to subsequently use these for protein prenylation (Ibrahim et al., 2001). These reports of protein prenylation in apicomplexan parasites suggest important requirements for isoprenoids in these pathogens. The apparent ability of both *Toxoplasma* and *Plasmodium* to salvage farnesyl and geranyl geranyl compounds could suggest a limited or non-essential role of the plastid-localized pathway in providing these compounds in apicomplexans. Obviously, this will heavily depend on the availability of these compounds in the infected host cell.

Dolichols: Sugar donors in apicomplexan parasites

Compounds with more than four isoprenoid precursors (5-22) are broadly categorized as polyisoprenoids. Polysioprenoids serve as cofactors in the synthesis of glycoproteins, glycosyl-phosphatidylinositol anchors, bacterial peptidoglycans, and are responsible for maintaining the fluidity of the cell membranes and can also act as donors for the prenylation of proteins. They can be divided into two main groups. Dolichols are isoprenoid alcohols found in all animal cells and some bacteria, protists,

fungi, and plants. Polyprenols are isoprenoid alcohols found mostly in plant tissues (D'Alexandri et al., 2006; Rip et al., 1985). *Plasmodium* parasites fed with radioactive GGPP, use this to synthesize dolichol. This dolichol was also used to prenylate proteins (Moura et al., 2001). Synthesis of such long chain prenyl compounds is mediated by the enzyme long chain prenyl synthase/trasferase and a homologue of the same is annotated in *Toxoplasma* and *Plasmodium* [TGGT1_107760, PFB0130w]. Homologues of the same enzyme have been characterized in other species. FPP can act as the template onto which isoprene units are to the desired length (Schenk et al., 2001). Dolichol can also serve as an activated precursor for the transfer of glucose and mannose residues in the secretory glycosylation pathway. In addition oligosaccharides are build up on a dolichol precursor before they are transferred en bloc to the N-glycosylation sites of secretory proteins. An enzyme catalyzing the synthesis of dolichol mannose was recently characterized in *Plasmodium*. Dolichol phosphate mannose is an important mannose donar for protein glycosylation (Shams-Eldin et al., 2008). A homologue of the same enzyme has been annotated in *Toxoplasma* (TGGT1_104570) and there have been several reports on the role of dolichol-conjugated sugars in the glycosylation of proteins and GPI type lipids in *Toxoplasma* (Striepen et al., 1999; Tomavo et al., 1992). The need for dolichol as an essential cofactor for glycosylation could potentially dictate the need for the apicoplast localized pathway to provide isoprenoid precursors for dolichol synthesis.

2.5.3 Isopentenyl tRNA's: Reading the apicoplast way.

Isoprenylated tRNAs for apicoplast genome translation

Despite the fact that the apicoplast lost the majority of its genome to the host through extensive gene transfer, it still maintains an organellar genome of approximately 35 kb size. This genome has been instrumental in identifying the organelle as a plastid. The reduced plastid genome primarily encodes for ribosomal proteins and tRNAs needed for protein translation. Electron micrographs of *T. gondii* (McFadden et al., 1996) and *P. falciparum* (Hopkins et al., 1999) apicoplasts show granular structures consistent with organellar ribosomes. Apicoplast genome expression has been studied in most detail in *P. falciparum* and the transcription appears to be polycistronic (Gardner et al., 1991a; Wilson et al., 1996). Interestingly many genes in the apicoplast genome appears to contain internal stop codons (Cai et al., 2003; Wilson et al., 1996), This also includes the gene coding for the RNA polymerase (Weiss, 2007). If the RNA polymerase is critical for the maintenance of apicoplast genome, how can the parasite tolerate a premature stop codon?

Internal stop codons in bacterial mRNAs can be read through with the help specialized tRNAs called stop codon suppressor tRNAs. This suppression of the stop codon can be a result of mutations in the tRNA, or tRNAs with unconventional base pairing (Urban and Beier, 1995). The tRNAs that have been shown to do the latter in bacteria are tRNAs carrying tryptophan with a CCA anticodon and tRNAs carrying cysteine with a GCA anticodon (Urban and Beier, 1995). Such unconventional base pairing is accomplished with the help of a modification of the nucleotide residues

adjacent to the first anticodon of the tRNA. One such modification is the isopentenylation of adenosine. This bulky and hydrophobic modification is thought to stabilize the intrinsically weak A–U base-pairing interaction between the first anticodon and its codon. The modification negates the need to recognize the third codon and this results in suppression and read through of the message (Ericson and Bjork, 1991; Hagervall et al., 1990; Petrullo et al., 1983; Urban and Beier, 1995). Importantly, this type of suppression has been identified in tRNAs encoded by chloroplast genomes in plants (Persson et al., 1994).

Isopentenylation of tRNA is mediated by the enzyme isopentenyl transferase, which transfers DMAPP to the amide moiety at position six of adenosine (i6A). This enzyme is also called miaA in *E. coli*. In bacteria, this modified tRNA is further methylthiolated by the enzyme miaB. The initial isopentenylation is required for this additional modification. This can be further hydroxylated by the enzyme miaE to yield ms2io6A (Persson et al., 1994). The genomes of *Toxoplasma* and *Plasmodium* encode enzymes involved in the modification process (Ralph et al., 2004). The tRNAs that could read codons starting with “U” (all stop codons start with U) are those that carry aminoacids Phe, Ser, Cy, Trp, Tyr and Leu. All these tRNAs are encoded in the apicoplast genome of *Toxoplasma* (Weiss, 2007), *Eimeria* (Cai et al., 2003) and *Plasmodium* (Preiser et al., 1995). These tRNAs could be modified using isoprenoid precursor to jump the stop codons during translation of the message transcribed by the apicoplast genome.

The apicoplast genome: What is it needed for?

The translation of the apicoplast genome is essential for the parasite as demonstrated by the surprising antiparasitic activity of antibiotics targeting the prokaryotic type translation machinery (Istvan et al.; Wiesner et al., 2008). Most of the apicoplast genome encodes proteins required for the transcription and translation of the genome. Only two protein coding genes have been attributed functions other than gene expression so far: sufB and clpC. SufB is an important enzyme involved in the generation of iron sulfur (Fe-S) clusters, and clpC is thought to be a chaperone component of the translocon complex involved in apicoplast protein import (Wilson et al., 2003). The ability of the apicoplast to generate Fe-S clusters and its importance was first documented with the identification of a plantlike Ferredoxin Nitrate reductase (FNR) enzyme of *Toxoplasma gondii* along with its reducing partner, a small Fe-S cluster containing protein ferredoxin (Vollmer et al., 2001) and its localization to the apicoplast (Striepen et al., 2000). Detailed biochemical characterization revealed that ferredoxin, which contains a critical Fe-S cluster acts as an important source of reducing potential for many of the enzymes in the apicoplast (Seeber, 2002). Apicoplast enzymes that require a functional Fe-S cluster are lipoic acid synthase, the last two enzymes of the DOXP pathway, and miaB. The conservation of the isoprenoid pathway among the members of the phylum could argue that the genome is maintained to encode the enzyme involved in Fe-S clusters assembly, as Fe-S clusters are needed for the DOXP pathway. Another potential requirement for the maintenance of the genome could be clpc's role in protein import

Isoprenylated tRNAs: A potential source of cytokinins

Isoprenylated adenosine can also give rise to cytokinins, important hormones that regulate the growth and development of plant cells. There has been considerable recent interest in a potential role of plant and algal hormones in apicomplexan biology recently (Nagamune et al., 2008b). Naturally occurring cytokinins are adenines carrying an isoprenoid or an aromatic side chain (Sakakibara, 2006). Two pathways are currently proposed for the synthesis of isoprenoid cytokinins. One is the direct pathway that results in the modification of 5'-AMP with the isoprenoid precursor DMAPP or HMBDP (Kakimoto, 2003). The second or indirect route involves the excision of modified adenosines from tRNAs carrying isopentenylated adenosine. Cytokinin activity from such modified tRNA was identified initially unexpectedly in tRNA from *E.coli* (Burrows et al., 1968; Hall, 1964; Skoog et al., 1966). The existence of this alternative pathway is further supported by the observationsin *Agrobacterium tumefaciens*. Despite deleterious mutations in the direct pathway for cytokinin biosynthesis *A. tumefaciens* releasea low amounts of free cytokinins into the culture medium (Kaiss-Chapman and Morris, 1976). Mutations in the indirect pathway e.g. inactivation of the miaA gene , abolished cytokinin secretion demonstrating a role of modified tRNAs in the synthesis of cytokinins (Gray et al., 1996). The potential of the apicoplast to generate isoprenylated tRNAs could mean that provides a source of cytokinins in the parasite. A function for cytokinins in parasite biology, if present, has yet to be established..

2.5.4: Apicoplast DOXP pathway and its suggested role in parasite egress

Death associated with apcioplast loss is often delayed. Parasites successfully undergo a single cycle of replication and host cell lysis before they cease development. This was observerd e.g. in plastid division mutants of *T. gondii* ,where the loss of the organelle did not prevent invasion and division, but resulted in failure to egress from the host cell (He et al., 2001). This could point to a specific role of the apicoplast in parasite host cell egress. A key trigger in the egress process is a change in the cellular calcium level. Elevated calcium levels have been implicated in the induction of parasite motility, invasion and egress (Black et al., 2000; Sibley, 2004). A mutant in *T. gondii* lacking a perforin-like has further implicated calcium levels in the regulation of the egress process (Kafsack et al., 2009). The factors controlling the levels of intracellular calcium still remain to be fully characterized in apicomplexan parasites. A potential role for the apicoplast in calcium regulation in *T. gondii* has been suggested through the synthesis of abscisic acid (Nagamune et al., 2008a). Abscisic acid is a hormone known to be involved in up regulation of intracellular calcium levels in plants. The *T. gondii* study showed that abscisic acid can stimulate the secretion of micronemes, a process important for motility that is dependant on increased intracellular calcium levels. Two pathways have been proposed for the synthesis of abscisic acid a direct pathway that operates in some plant pathogenic fungi where abscisic acid is derived from FPP and an indirect pathway found in plants that occurs through the cleavage of carotenoids (Schwartz et al., 2003). *T. gondii* is sensitive to fluoridone, a herbicide that inhibits the indirect pathway and this finding could implicate the indirect pathway as a potential

route for abscisic acid production in apicomplexans. The pathway is initiated by the formation of beta-carotene, a long chain isoprenoid compound containing eight isoprenoid precursors and the subsequent degradation of it through sequential enzymatic reactions. Initial genomic analysis failed to identify the homologues of the enzymatic machinery in this indirect pathway (Nagamune et al., 2008b), however the responsiveness of the parasite to abscisic acid and its sensitivity to fluoridone suggests that further effort to identify those enzymes may be well invested.

2.5.5 Mitochondria and Apicoplast: Partners in Parasitism

Ubiquinone prenylation: Potential link between apicoplast and mitochondria

Most apicomplexan parasites have a single tubular mitochondrion (Mather and Vaidya, 2008), while *Cryptosporidium* has a reduced mitochondria-like structure referred to as mitosome (Keithly et al., 2005). There have been reports of a close physical association of mitochondrion and apicoplast in apicomplexans that indicates a potential role of the apicoplast in maintaining mitochondrial homeostasis (or vice versa). Some support for this idea came from a study that ablated the apicoplast phosphate transporter in *T. gondii* (Brooks et al., 2010). This membrane transporter imports metabolites to support the pathways in the apicoplast. Mutation of the apicoplast transporter resulted in a subsequent loss of the mitochondrial membrane potential. This loss of mitochondrial membrane potential was not seen with the ablation of fatty acid biosynthetic pathway in the apicoplast. This argues that the effect is due to the loss of other apicoplast pathways, most likely the isoprenoid biosynthesis pathway. A potential

link between the mitochondrion and the isoprenoid pathway in the apicoplast is the requirement for isoprenoid precursors for the synthesis of ubiquinone. Ubiquinone is an essential part of mitochondrial electron transport chain and critical to maintain a membrane potential. Along the same lines, fosmidomycin, has been shown to reduce ubiquinone levels in *Plasmodium* (Cassera et al., 2007). Ubiquinone biosynthesis takes place in the mitochondrion with 4-hydroy benzoate acting as the precursor for the synthesis, which is prenylated in the first step of the biosynthetic process. A likely candidate enzyme involved in the transfer of prenyl chain has been identified in *Toxoplasma* and *Plasmodium* [TGGT1_010780, PFF0370w]. Apicoplast synthesized IPP may be required for the synthesis of a specific chain length isoprenoid to prenylate ubiquinone. The length of ubiquinon prenyl side chain varies between species (Tonhosolo et al., 2005) and a suitable isoprenoid may not be available in the host cell.

Functional importance of ubiquinone and mitochondria in apicomplexan parasites

Death associated with this loss of the apicoplast transporter in *T. gondii* was swifter than death associated with the loss of just the apicoplast fatty acid pathway (Brooks et al., 2010; Mazumdar et al., 2006). A trivial explanation could be that since the transporter supplies two plastid pathways its loss may be costlier than the loss of fatty acid synthesis alone. The fact that loss of the apicoplast transporter resulted in loss of mitochondrial membrane potential might argue otherwise. Isoprenoid synthesis which is also fed by APT might have a more direct and more deadly effect on the mitochondrion.

The requirement for oxidative phosphorylation and ATP production in the mitochondrion if apicomplexans has been the topic of lively discussion. The absence of a mitochondrial pyruvate dehydrogenase capable of converting pyruvate to acetyl-CoA argues for an incomplete or even inactive TCA cycle (Foth et al., 2005; Olszewski et al., 2010). Furthermore some critical components of the ATP synthase may be absent in both *Plasmodium* and *Toxoplasma* (Gardner et al., 2002; Mather and Vaidya, 2008)

Plasmodium blood stages primarily use glycolysis to supply ATP, while retaining some components of the electron transport chain in mitochondria (Olszewski et al., 2010). There have been conflicting reports about the ability of *Plasmodium* mitochondrion to perform oxidative phosphorylation. While some studies suggest that *Plasmodium* mitochondria are not a source of ATP (Fry et al., 1990), others report oxidative phosphorylation in both *P. berghei* and *P. yoelli*. In a study iusing genetic and pharmacological approaches researchers showed that the most important function of mitochondria in *Plasmodium* might be to act as an electron sink for the pyrimidine biosynthetic process involving dehydroorotate dehyrdrogenase (Painter et al., 2007; Vaidya et al., 2008). Pyrimidine synthesis is essential for the parasite (Gutteridge et al., 1979). Importantly this ability of the mitochondrion depends on ubiquinone which accepts the electrons. The *Toxoplasma* mitochondrion on the other hand has been demonstrated to engage in mitochondrial oxidative phosphorylation (Mather and Vaidya, 2008; Vercesi et al., 1998). This is further supported by a recent study which showed that a drug that specifically targets mitochondrial dehydrogenase also had an effect on cellular ATP levels in *Toxoplasma* (Lin et al., 2009). In any case, ubiquinone is essential

for both oxidative phosphorylation and pyrimidine biosynthesis,. Loss of the apicoplast pathway to supply the isoprenoid precursors for ubiquinone biosynthesis could be the reason for the loss of mitochondrial membrane potential. Latly, *Toxoplasma* and *Plasmodium* are sensitive to the drug atovaquone, a structural analogue of ubiquinone that targets the mitochondrial electron transport chain (Baggish and Hill, 2002). This argues for a vital role of ubiquinone and at least part of the mitochondrial electron chain in both organisms.

How was ubiquinone prenylated prior to the endosymbiosis that let to the apicoplast? The host organism must have had its own pathway to synthesize the isoprenoid precursors, most likely a mevalonate pathway as found in ciliates (a related non photosynthetic alveolate). This ability was apparently subsequently lost. By metabolic streamlining photosynthetic progenitor of apicomplexans came to rely solely on the pathway of its symbiont.

Heme a prenylation: Another potential link between apicoplast isoprenoid synthesis and mitochondria

Biosynthesis of heme is a shared responsibility between the apicoplast and the mitochondrion. The heme biosynthetic pathway starts and terminates in mitochondria generating functional heme, while the intermediates of the pathway shuttle between the apicoplast and the mitochondrion (Ralph et al., 2004). The major function of the heme generated in the mitochondria is to serve as prosthetic group for enzymes in the electron transport chain called cytochromes. Although there have been reports on the

parasites' ability to synthesize heme de novo (Surolia and Padmanaban, 1992), whether this process is essential requires further investigation There have been reports to indicate that the heme associated with cytochrome a has a long isoprenoid side chain, mostly a farnesyl chain (Schafer and Rine, 1992; Weinstein et al., 1986) which could also be supplied by the apicoplast isoprenoid pathway.

2.6 Conclusions

Out of the three metabolic pathways of the apicoplast, the functional needs of the fatty acid pathway have been studied most extensively in both *Toxoplasma* and *Plasmodium*. The pathway is essential for survival and needed for apicoplast biogenesis in *Toxoplasma* (Mazumdar et al., 2006). *Plasmodium* needs the pathway for liver stage development, but can live without it during red blood cell stage and the insect stage (Vaughan et al., 2009; Yu et al., 2008). *Eimeria* also has a functional FASII pathway (Lu et al., 2007; Mazumdar and Striepen, 2007), but genome analysis using bioinformatic tools suggest that the pathway is absent in the apicoplast of *Babesia* and *Theileria* (Brayton et al., 2007; Lau, 2009). *Toxoplasma*, *Plasmodium* and *Eimeria* also have other systems to make fatty acids, an elongase system associated with the ER as well as cytoplasmic FASI system (Lu et al., 2007; Mazumdar and Striepen, 2007). Such redundancy argues that retention of this organelle may as well be for some other critical functional needed during evolution. The other important pathway hosted exclusively by the apicoplast is the one that makes isoprenoid precursors IPP and DMAPP. This unique pathway is essential for all the stages of *Plasmodium* (Odom and Van Voorhis,

2010). Genome analysis reveals that enzymes involved in the isoprenoid precursor pathway are present in all the apicoplast harboring apicomplexans (Brayton et al., 2007; Clastre et al., 2007). [Fig 2.5] summarizes our current (and speculative) view of the likely evolution of plastid pathways in apicomplexans., It is our hypothesis that the isoprenoid pathway is the most important function of the apicoplast and we will provide some experimental evidence for this idea in the next chapter.

Figure 2.1. Catalytic reactions of apicoplast localized isoprenoid biosynthesis pathway; substrates of the pathway; DOXP, 1-deoxy-D-xylulose-5phosphate; MEP, 2C-methyl-D-erythritol-4-phosphate; CDP-MEP, cytosine dinucleotidephosphate 2C-methyl-D-erythritol; CDP-MEP cytosine dinucleotidephosphate 2C-methyl-D-erythritol phosphate; MEcPP, methyl erythritol cyclopyrophosphate; HMBPP, hydroxymethy-but-enyl pyrophosphate; IPP,Isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate. Enzymes in the pathway are italicized. *doxps,* DOXP synthase; *doxpri*, DOXP reductoisomerase; *ispD,* 4-Diphosphocytidyl-2C-methyl-D-erythritol-4-phosphate synthase; *ispE,* 4-Diphosphocytidyl-2C-methyl-D-erythritol kinase; ; *ispF*, 2C-methyl-D-erythritol-2, 4- cyclodiphosphate synthase; *ispG*, 1-hydroxy-2-methyl-2-(E)-butenyl- 4-diphosphate synthase. *lytB,* 1- hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase.

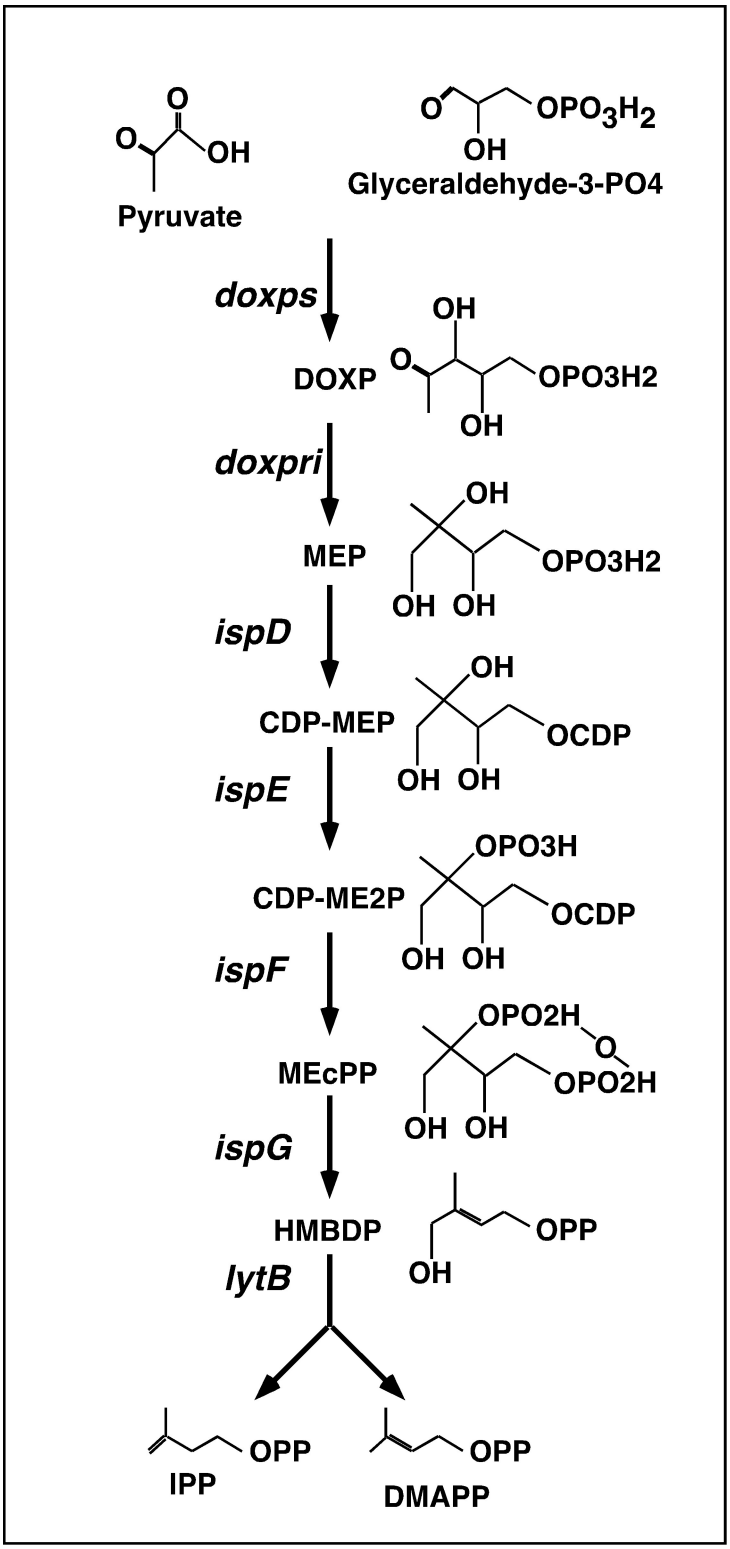

Pyruvate
Glyceraldehyde-3-PO4
doxps
DOXP
doxpri
MEP
ispD
CDP-MEP
ispE
CDP-ME2P
ispF
MEcPP
ispG
HMBDP
lytB
IPP
DMAPP

Figure 2.2: Fluorescent growth assay of *T.gondii* testing drugs targeting the apicoplast localized DOXP pathway A. Fluoroscent growth assay showing that *Toxoplasma* is resistant to FR-900098 , a methyl derivative of fosmidomycin that inhibits *dxpri*. **B.** Fluoroscent growth assay showing that Ketoclomazone, a drug that targets the enzyme *doxps* of apicoplast isoprenoid synthesis pathway is effective at higher concentrations.

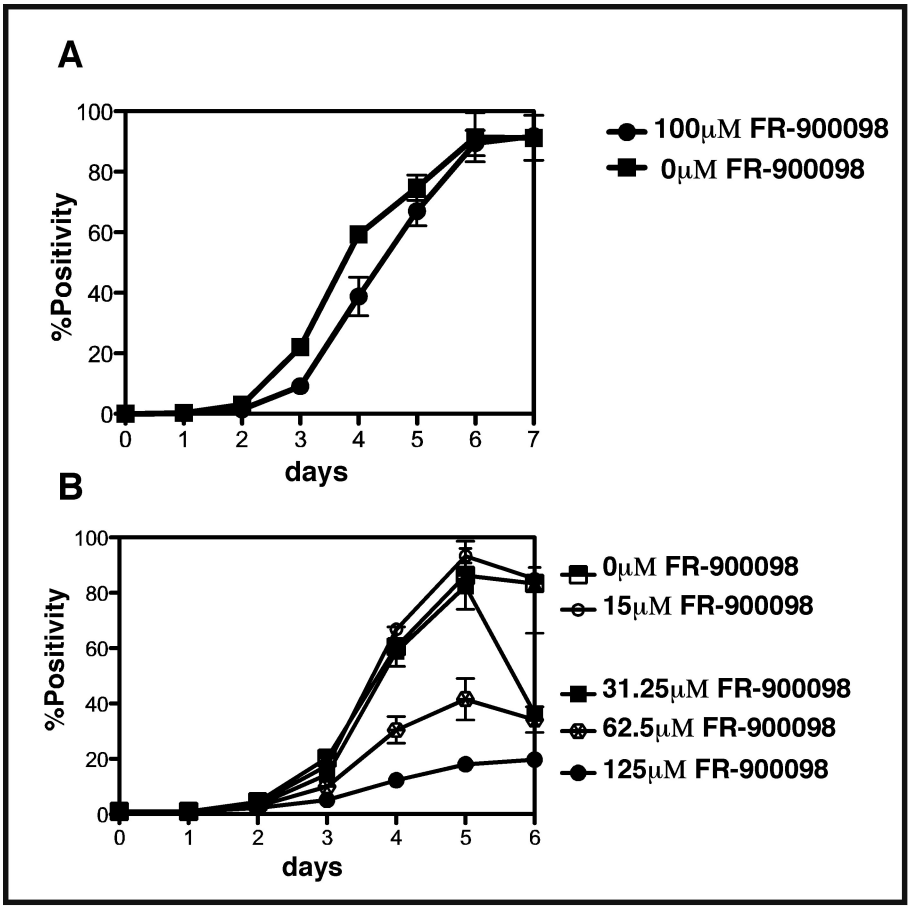


Figure 2.3: Schematic representation of the barriers that fosmidomycin need to cross to get into the apicoplast lumen:

Abbreviations used: Fosm, Fosmidomycin; PV, Parasitophorous vacuole

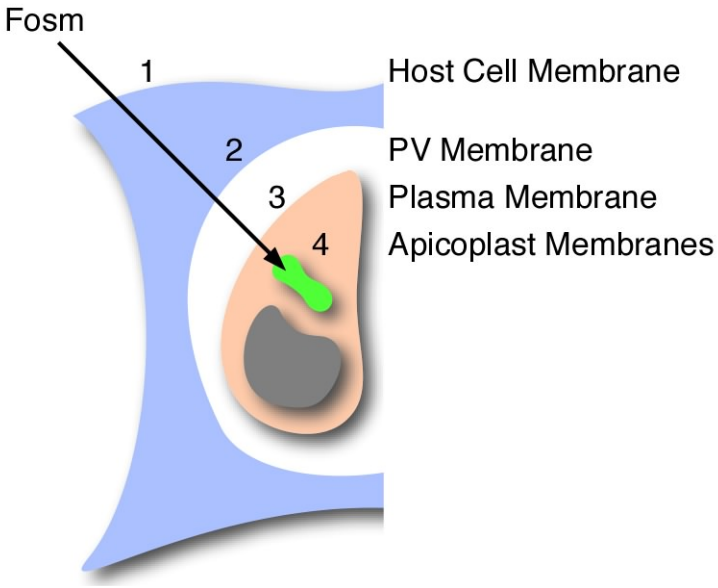


Figure. 2.4. Isoprenoid precursor biosynthesis pathway and its functional needs in Apicomplexa. Inhibitors targeting the specific catalytic step are also shown. Enzyme abbreviations;mia A/B/E, tRNA isopentenylation enzymes; GPPS, Geranyl pyrophosphate synthase; FPPS, Farnesyl pyrophosphate synthase; GGPPS, Geranyl geranyl pyrophosphate synthase. ThiF complex: Includes ThiF,ThiS,ThiI,IscS,ThiG,ThiH/ThiO ;TPK, Thiamine pyrophosphate kinase

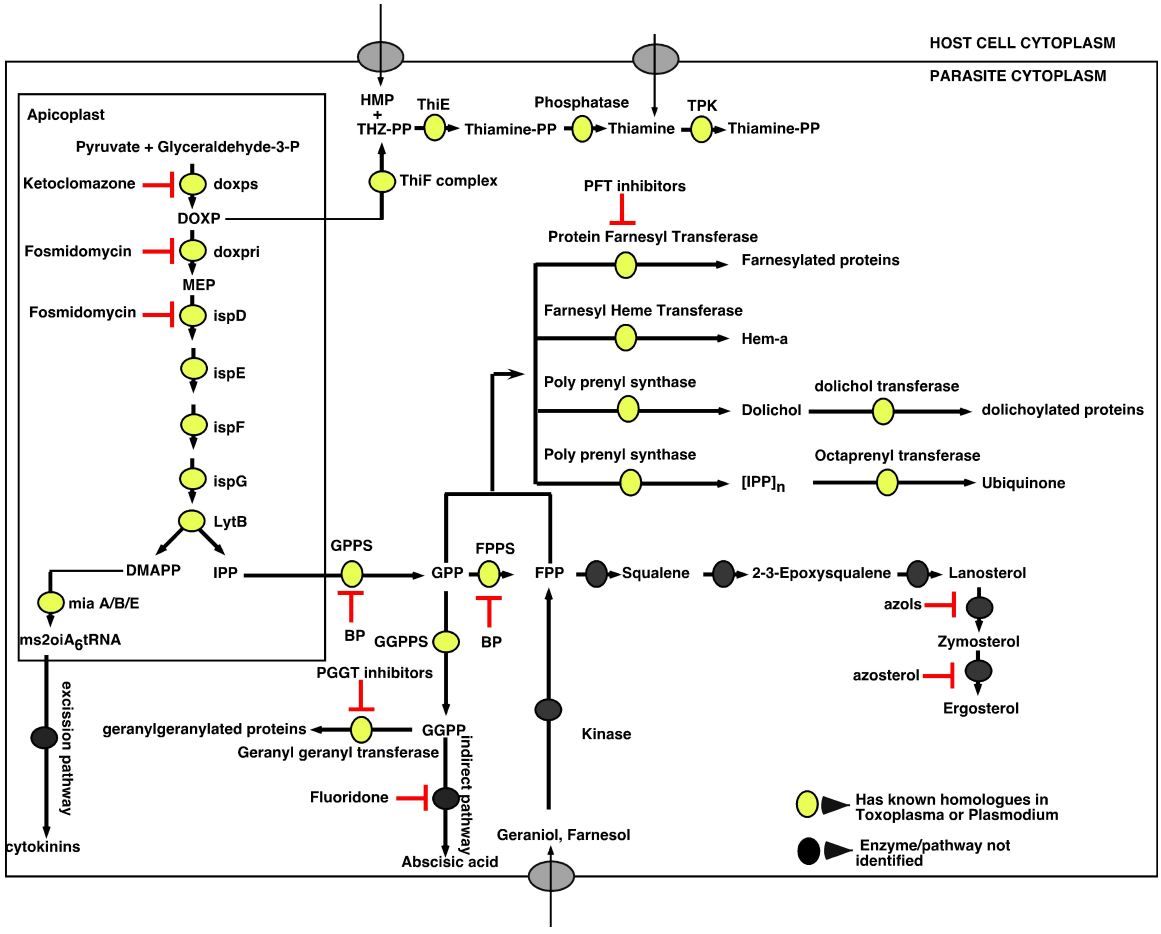
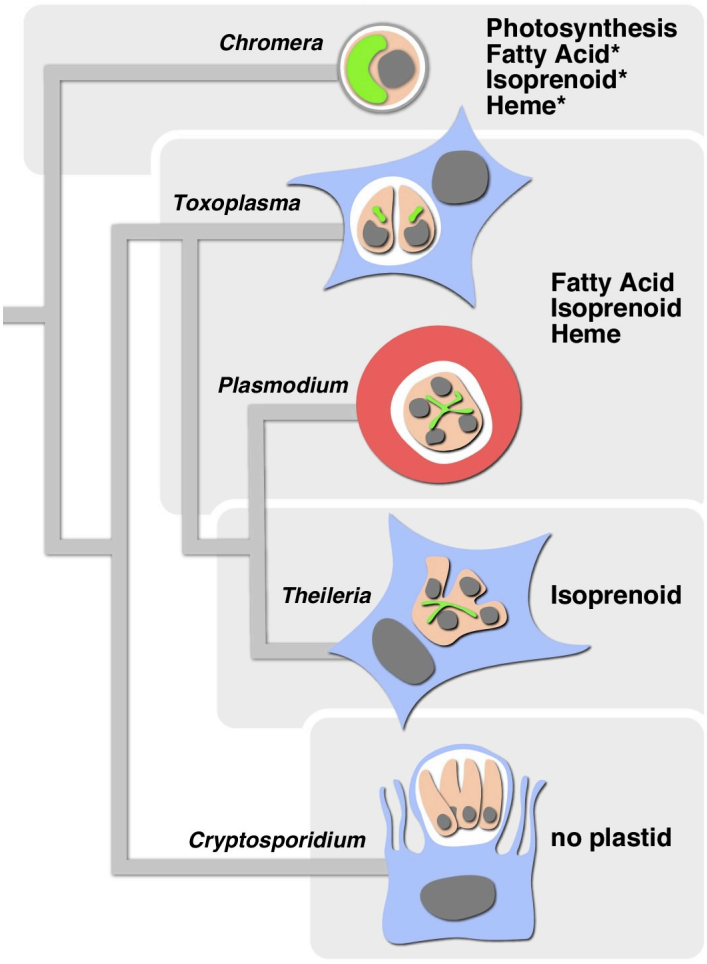


Figure.2.5: Cartoon showing conservation of isoprenoid pathway across the phylum. *Chromera*, the photosynthetic ancestor of apicomplexa along with *Toxoplasma* and *Plasmodium* has all the three pathways in the apicoplast, while *Theileria* and *Babesia* have the isoprenoid pathway only indicating conservation across the phylum



CHAPTER 3

APICOPLAST ISOPRENOID PRECURSOR SYNTHESIS AND THE MOLECULAR BASIS OF FOSMIDOMYCIN RESISTANCE IN TOXOPLASMA GONDII

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doi: 10.1084/jem.20110039, reprinted with permission

3.1. ABSTRACT

Apicomplexa are important pathogens that include the causative agents of malaria, toxoplasmosis, and cryptosporidiosis. Apicomplexan parasites contain a relict chloroplast, the apicoplast. The apicoplast is indispensable and an attractive drug target. The apicoplast is home to a 1-deoxy- D -xylulose-5-phosphate (DOXP) pathway for the synthesis of isoprenoid precursors. This pathway is believed to be the most conserved function of the apicoplast, and fosmidomycin, a specific inhibitor of the pathway, is an effective antimalarial. Surprisingly, fosmidomycin has no effect on most other apicomplexans. Using *Toxoplasma gondii* , we establish that the pathway is essential in parasites that are highly fosmidomycin resistant. We define the molecular basis of resistance and susceptibility, experimentally testing various host and parasite contributions in *T. gondii* and *Plasmodium*. We demonstrate that in *T. gondii* the parasite plasma membrane is a critical barrier to take up drug . In strong support of this hypothesis, we engineer de novo drug-sensitive *T. gondii* parasites by heterologous expression of a bacterial transporter protein. Mice infected with these transgenic parasites can now be cured from a lethal challenge with fosmidomycin. We propose that the varied extent of metabolite exchange between host and parasite is a crucial determinator of drug susceptibility and a predictor of future resistance

Abbreviations used: APT, apicoplastphosphate translocator; ATc, anhydrotetracycline; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy- d -xylulose-5-phosphate;

DOXPRI, DOXP reductoisomerase; FASII, type II fattyacid synthesis; G3P, glycerol-3-phosphate; GlpT, G3P transporter;IPP, isopentenyl pyrophosphate.

Key words: *Toxoplasma*, apicoplast, isoprene, malaria, drug resistance

3.2. INTRODUCTION

Apicomplexan parasites are the cause of many important infectious diseases. These include malaria, toxoplasmosis, and cryptosporidiosis in man and theileriosis, babesiosis, and coccidiosis in domestic animals. At this point, no effective vaccines are available to prevent diseases caused by apicomplexans in humans, and management therefore relies on drug treatment. Treatment of malaria, in particular, is threatened by the rapid emergence of drug-resistant parasites, and a constant stream of new drugs with new modes of action is required to stay ahead of the pathogen. One of the most promising sources for new targets is the apicomplexan plastid or apicoplast (Wiesner et al., 2008). The apicoplast is believed to be the remnant of an alga and the product of an ancient secondary endosymbiosis that gave rise to the super phylum Chromalveolata (Gould et al., 2008). Numerous chromalveolate groups, such as kelps or diatoms, are still photosynthetic, whereas others like ciliates and select apicomplexans, have lost their photosynthetic ability and have turned to predation or adopted parasitism. Despite the loss of photosynthesis, the apicoplast is critical for the survival of apicomplexans. Pharmacological and genetic studies in *Toxoplasma gondii* and the malaria parasite *Plasmodium* have shown that loss of the apicoplast, loss of its genome, or loss of its metabolic function result in the death of these parasites. The overall picture that has emerged over the last decade is that the apicoplast functions similarly to a chloroplast in the dark in that it acts as an anabolic hub providing metabolites for the parasite cell (Ralph et al., 2004; Seeber and Soldati-Favre, 2010). Three major anabolic pathways

have been identified in the apicoplast: a type II fatty acid synthesis (FASII) pathway, a 1-deoxy- d -xylulose-5-phosphate (DOXP) pathway for the synthesis of isoprenoid precursors, and a portion of the heme biosynthetic pathway. Different apicomplexan parasites exhibit marked differences as to which of these pathways are present. The most conserved metabolic function of the apicoplast appears to be the DOXP pathway, as the genes encoding the enzymes for isoprenoid synthesis are thus far found in all apicomplexans that harbor an apicoplast.

The DOXP pathway was first discovered in the chloroplasts of plants and algae then subsequently identified in eubacteria (Horbach et al., 1993 ; Lichtenthaler et al., 1997). The pathway is also referred to as the nonmevalonate pathway to highlight its use of an entirely different set of enzymes from the classical mevalonate pathway used by animals and fungi. Despite starting from different substrates, both the DOXP and the mevalonate pathway converge on one end product isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are precursors used to synthesize a wide variety of lipids with important functions in membrane structure, protein modification, metabolism, and hormone signaling including cholesterol, dolichol, farnesol, abscisic acid, ubiquinone, modified tRNAs, and isopentenylated proteins.

Apicomplexa lack a mevalonate pathway but harbor a DOXP pathway in the apicoplast. Because the parasite DOXP pathway is mechanistically different from the host mevalonate pathway, it appears to be a promising drug target in apicomplexans, and fosmidomycin—an antibiotic which specifically inhibits DOXP reductoisomerase

(DOXPRI)—shows robust activity against *Plasmodium* and *Babesia* in vitro and in animal models (Jomaa et al., 1999). Fosmidomycin is also effective in the clinical treatment of uncomplicated cases of malaria when combined with other drugs that target apicoplast functions (Lell et al., 2003). Surprisingly, though, fosmidomycin has no eff ect on the growth of many other members of the phylum including *Eimeria* , *Theileria* , and *T.gondii* (Ling et al., 2005 ; Clastre et al., 2007 ; Lizundia et al.,2009). Nevertheless, genome mining identifies genes encoding the enzymes of the DOXP pathway in these fosmidomycin-insensitive organisms, and expression studies suggest that the genes are actively transcribed (Clastre et al., 2007 ; Moreno and Li, 2008). One rationalization for such differential sensitivity is that apicoplast isoprenoid synthesis might not be as important for parasite survival as anticipated.

Indeed, not all biosynthetic functions of the apicoplast are essential over the entire parasite life cycle. The synthesis of fatty acids that occurs in the apicoplast in parallel to the synthesis of isoprenoid precursors has been studied in more detail and the findings illustrate this point. Genetic studies revealed that apicoplast fatty acid synthesis is essential for *T. gondii* and the initial liver stages of *Plasmodium*, but surprisingly, this pathway was found to be dispensable in the subsequent blood phase of the malaria parasite (Mazumdar et al., 2006 ; Yu et al., 2008 ; Vaughan et al., 2009). Apparently the erythrocytic stage of the parasite relies entirely on the salvage of fatty acids and this is not feasible or sufficient in other host cell niches. This suggests that the importance of a particular anabolic pathway depends on the specific host cell infected by the parasite and that; therefore, the mere presence of the enzymes does not prove

their essentiality. Fosmidomycin appears to be highly effective against apicomplexans that develop within RBCs like *Plasmodium* and *Babesia*. *Babesia* causes a disease similar to malaria in cattle and a variety of other mammals. In contrast, those apicomplexans that are resistant for fosmidomycin parasitize nucleated cells; *Theileria* infects lymphocytes, *Eimeria* infects epithelial cells, and *T. gondii* develops in macrophages and many other nucleated cell types. One hypothesis could therefore be that isoprenoids are a limiting resource in RBCs but can be salvaged from nucleated cells that may more vigorously synthesize these compounds and, thus, render synthesis by the parasite redundant.

The hypothesis that parasitism of nucleated cells allows isoprenoid salvage is contrary to our interpretation of recent experiments tracing the carbon flux into the apicoplast (Brooks et al.,2010; Lim et al., 2010). In these studies, we constructed a conditional mutant in the apicoplast phosphate translocator (APT) of *T. gondii* . We demonstrated that blocking the metabolic activity of the apicoplast by ablating carbon import through APT results in the rapid demise of the parasite. We further showed that carbon import is required for fatty acid synthesis as expected but, more importantly, that blocking import had a more pronounced growth effect than blocking just FASII. We took these results as an indication that the DOXP pathway, which like FASII is fed by APT (Fig 3.1), should be essential in *T. gondii* and, hence, a promising drug target. This view is obviously not supported by the lack of activity of fosmidomycin. In this study, we use *T. gondii* as a model organism to resolve this conundrum and make comparisons with *Plasmodium* in different host cells. We provide unequivocal genetic evidence for

the importance of the apicoplast isoprenoid pathway. We explore several potential molecular mechanisms for fosmidomycin resistance and conclude that *T. gondii* is resistant as the result of a lack of drug uptake at the level of the parasite cell membrane by engineering a de novo drug-sensitive parasite strain.

3.3. MATERIALS AND METHODS

3.3.1. Parasite culture and growth assays.

RH strain *T. gondii* tachyzoites were maintained in human fibroblasts and genetically manipulated as detailed previously (Striepen and Soldati, 2007). Parasite growth was measured by fluorescence assay (Gubbels et al., 2003). This assay quantifies parasite fluoresecence daily for a week in a 96-well format. Parasites are fluorescent because of expression of either a tandem YFP (Fig. 3.1 and 3.7) or a tandem tomatoRFP (Fig. 3.2 and 3.3) transgene. Drug assays were performed by twofold serial dilutions of drug using concentrations ranging from 100 to 0.375 µM. IC 50 values were calculated with Prism software (GraphPad Software) using a nonlinear dose-response curve to fit normalized fluorescence values from day 4 (the phase of exponential growth). Fosmidomycin was provided by H. Jomaa (Institut für Klinische Chemie und Pathobiochemie, Justus-Liebig- Universität Giessen, Giessen, Germany and Y. Song). All experiments were conducted at least in duplicate.

Coverslip cultures were seeded with HepG2 cells and infected with P. berghei ANKA sporozoites dissected from the salivary glands of infected Anopheles

stephensi (Schmidt-Christensen et al., 2008). 10 µM fosmidomycin was added to the media 12 h after infection and medium was changed every 24 h. Infected cells were incubated at 37°C and 8% CO 2 until fixation for IFA (50 h after infection) or until infected cells detached and formed merosomes (65 h).

3.3.2. Genetic manipulations.

5' RACE PCR experiments were performed using the RACE kit (BD). Fig. S1 shows a detailed schematic overview of the genetic manipulations used and Tables 3.1, 3.3, and 3.4 provide all primers used. The validated or corrected coding sequences were amplified and introduced into plasmid pDT7S4myc3 (see Tables 3.2 and 3.3 for specific primers and restriction sites), placing them under the control of a tet-regulated promoter as detailed previously (Brooks et al., 2010). Clonal lines were established in a transactivator strain (Meissner et al., 2002) by transfection and pyrimethamine selection. Gene-targeting constructs were derived from cosmid PSBLA92 (LytB) or TOXP686 (DOXPRI) by recombineering in E. coli EL250 (Brooks et al., 2010). These modified cosmids were then used to disrupt the native loci by double homologous recombination and replacement of the entire coding region with chloramphenicol acetyl transferase, and stable clones were derived by chloramphenicol selection. Deletion was confirmed by PCR and Southern blotting. Genomic parasite DNA was restricted as indicated, separated on 0.7% agarose gels, and transferred to nylon membrane. Probe DNA was prepared by PCR (Fig. S2 provides maps indicating the positions of probes and restriction sites) and ^{32}P labeled by random priming. For complementation analysis,

coding sequences were introduced into plasmid pBTTy placing them under control of the constitutive tubulin promoter (Brooks et al., 2010) and stable transformants were established by phleomycin selection (Soldati et al., 1995). To facilitate fluorescence growth assays, parental, mutant, and complemented lines were transfected with plasmid PCTR 2T (van Dooren et al., 2008). Red fluorescent parasites expressing tandem tomato RFP were isolated by cell sorting using a MoFlo sorter (Dako).

3.3.3. Expression of *T. gondii* DOXPRI in E. coli.

The coding sequence for DOXPRI lacking the presumptive leader and transit peptide (aa 187–632) was cloned into expression plasmid pAVA0421, and the construct was introduced into E. coli BL21. Recombinant protein was purified under denaturing conditions and used to immunize rabbits (Cocallico Biologicals). We also expressed *T. gondii* DOXPRI (either aa 187–632 or aa 20–632) fused to the C terminus of maltose binding protein (New England Biolabs, Inc.). Enzymatic activity of purified MBP-TgDOXPRI was measured after the oxidation of NADPH to NADP + (Dhiman et al., 2005). We did not detect activity for the *T. gondii* recombinant enzyme but noted robust activity for the E. coli enzyme expressed in parallel.

3.3.4. Expression of *E. coli* DOXPRI in T. gondii.

We engineered a plasmid to target heterologous proteins to the apicoplast using the leader sequence of T. gondii ferredoxin NADP reductase (FNR, aa 1–150) and a Ty-1 epitope (pBTTYFNR$_L$). We amplified the coding sequence of E. coli DOXPRI

(AP000833) from strain DH5α and introduced it into pBTTYFNR$_L$. ΔDOXPRI parasites were transfected with the resulting plasmid and stable transformants were selected using phleomycin.

3.3.5. Expression of *E. coli* GlpT in T. gondii.

The coding sequence of the *E.coli* GlpT (NP416743) was amplified and cloned in frame with the full apicoplast leader sequence of FNR (pBTTYFNR$_L$ E.c.GlpT) or the signal sequence portion (pBTTYFNR$_{SP}$ E.c.GlpT). Point mutantions were introduced using QuickChange site-directed mutagenesis (Agilent Technologies). Mutagenesis was performed in plasmid PCR2.1-GlpT. Mutagenic primers were designed to introduce arginine to lysine changes (ACG to AAA) in GlpT at positions 45 and 269. Mutations were confirmed by sequencing and modified coding sequences were introducted into pBTTYFNR$_L$.

3.3.6. Immunofluorescence and Western blotting.

Immunofluorescence and Western assays were performed as described previously (Brooks et al., 2010). Primary antibodies were were used at 1:100 (c-myc; Roche), 1:5 (Ty-1; K. Gull, Oxford University, Oxford, England, UK), and 1:1,000 (anti–T. gondii DOXPRI, ACP [Waller et al., 1998], and Cpn60). Slides were viewed on a DMIRBE (Leica) or a DeltaVision microscope (Applied Precision). Images were captured, deconvolved, and adjusted for contrast using Openlab, Volocity, or Softworx

software (PerkinElmer or Applied Precision). For Western blots, primary antibodies were used at 1:100 (c-myc), 1: 2,500 (anti-DOXPRI), or 1:10 (Ty-1).

3.3.7. Parasite fosmidomycin uptake assay.

Samples were spotted onto 7-mm paper discs, and discs were place onto LB plates inoculated with 500 µl of an overnight culture of E. coli DH5α. Plates were incubated for 24 h at 37°C, scanned, and clear zones were measured. A standard curve was derived from a series of known drug amounts using a nonlinear model in Prism software. For uptake assays, parasites were grown in medium without antibiotics and serum to ~ 80% lysis. Parasites were released from host cells, filtered, and counted. 10^8 parasites were incubated for 6 h at 37°C in medium containing 100µM fosmidomycin. Subsequently parasites were pelleted, washed in PBS, repelleted, and suspended in 100µl of distilled water before sonication. 20µl of this lysate was loaded onto paper discs. All experiments were conducted at least in duplicate. Digitonin permeabilization.10^8 fosmidomycin-loaded parasites were resuspended in 50µl 0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA and mixed with the same buffer containing 0.2% digitonin (Esseiva etal., 2004). Cells were incubated on ice for 5 min before centrifugation. The pellet was disrupted by sonication. Specificity was tested using the FNRRFP/YFP-YFP parasite strain (L. Sheiner, University of Georgia, Athens,Georgia). Parasites were incubated as described in the previous section beforeadditon of excess medium followed by fluorescence microscopy or flow cytometry(Striepen and Soldati, 2007).

3.3.8. Synthesis of fosmidomycin derivatives.

Three esters of fosmidomycin were synthesized largely according to published methods (Reichenberg et al., 2001 ; Ortmann et al., 2003). The identities of these compounds were confirmed with ^{1}H and ^{31}P NMR using a 400-MR (Varian), and the respective purities were determined to be >95% by HPLC using a Prominence system (Shimadzu) fitted with a 4.6 × 250–mm Zorbax C18 column (Agilent Technologies). The following is a list of nuclear magnetic resonance (NMR) chemical shift coordinates: diphenyl-fosmidomycin, NMR shows the product exists as two rotamers with a ratio of ~7:3; ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.32 and 7.92 (s,1 H), 7.36–7.30 (m, 4H), 7.22–7.12 (m, 6H), 3.74–3.69(m, 2H), and 2.21–2.08 (m, 4H); ^{31}P NMR (162 MHz, $CDCl_3$), 28.8; di-4methoxyphenyl-fosmidomycin, two rotamers with a ratio of ~ 6:4; 1 H NMR (400 MHz, $CDCl_3$), 8.32 and 7.88 (s, 1H), 7.08–7.01 (m, 4H), 6.85–6.80 (m, 4H), 3.76 (s, 6H), 3.74–3.64 (m, 2H), and 2.18–2.02 (m, 4H); ^{31}P NMR (162 MHz, $CDCl_3$), δ 28.8; dipivaloxymethyl-fosmidomycin, two rotamers with a ratio of ~ 6:4; 1 H NMR (400 MHz, $CDCl_3$), 7.90 and 7.51 (s, 1H), 5.25–5.22 (m, 4H), 3.20–3.11 (m, 2H), 1.61–1.42 (m, 4H), and 0.82–0.84 (m, 18H); and ^{31}P NMR (162 MHz, $CDCl_3$), δ 36.6 and 37.1.

3.4. RESULTS

3.4.1. *T. gondii* is resistant to fosmidomycin, but the DOXP pathway is nonetheless essential.

The DOXP pathway is initiated by the condensation of pyruvate and glyceraldehyde-3-phosphate and consists of seven catalytic reactions leading to the synthesis of IPP and DMAPP (Fig. 3.1 A). Fosmidomycin is an inhibitor of the second and committing step that is catalyzed by the enzyme DOXPRI. Fosmidomycin mimics the substrate DOXP (Fig. 3.1 A) and binds to the active site of the enzyme (Steinbacher et al., 2003). Previous studies using *Eimeria* and *T. gondii* reported resistance of both parasites to fosmidomycin (Ling et al., 2005; Clastre etal., 2007). We confirmed this result. As shown in Fig. 3.1 B, *T. gondii* growth is normal in the presence of 100 µM of the drug. For comparison, the IC_{50} for *Plasmodium falciparum* and *Babesia bovis* are 0.4–1.2 and 3–4 µM, respectively (Jomaa et al., 1999; Sivakumar et al., 2008). *T. gondii* has been shown to take up isoprenoids in radiolabeling experiments (Ibrahim et al., 2001), and the DOXP pathway may therefore be dispensable. To test this hypothesis, we constructed a conditional mutant in the *T. gondii* LytB gene. LytB encodes the enzyme 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase that produces IPP and DMAPP in the final step of the DOXP pathway (Fig. 3.1 A). A candidate gene for LytB is readily identified in the *T. gondii* genome by similarity searches (toxoDB gene ID TGGT1_082920). The gene appeared not to encode an N-terminal signal peptide as typical for apicoplast proteins, but we note the presence of a potential recessed signal peptide at aa 156–179 (a similar presumptive recessed signal is present in the *P.*

falciparum homologue). We evaluated the gene prediction using 5' RACE PCR, and the sequence of the LytB transcript established in our experiments matched the predictions of gene model TGGT1_082920. We next engineered an expression construct that fused the LytB coding sequence to a C-terminal epitope tag and placed it under the control of a tetracycline regulatable promoter (see Fig. 3.9 for a schematic outline of the genetic strategy and further detail on the various selection markers and promoters used). The construct was transfected into a *T. gondii* transactivator strain (Meissner et al., 2002). Immunofluoroscence staining for the epitope tag was indistinguishable from that of the known apicoplast protein ACP, and the protein was proteolytically processed as typical for apicoplast proteins (Fig. 3.2, A and C). To establish whether apicoplast IPP synthesis is dispensable, we targeted the native LytB locus in this strain using a recently developed cosmid-based approach (Brooks et al., 2010). Successful gene deletion was confirmed by PCR and by Southern blotting (Fig. 3.2 B and Fig. 3.10 A). In this mutant strain, the epitope-tagged transgene is the only LytB gene, and its expression can be repressed with anhydrotetracycline (ATc). Western blot analyses show that after 2 d of treatment with ATc, LytBmyc is no longer detectable (Fig 3.2 C). We introduced a red fluorescent protein expression cassette into mutant (Δ LytB) and parental strain (iLytB) and measured parasite growth by plaque and fluorescence assay (Gubbels et al., 2003). Mutant parasites show a severe growth defect in the presence of ATc that was not observed in the parental strain. This phenotype could be fully rescued by complementing with a LytB gene under a constitutive α-tubulin promoter, suggesting that loss of growth is specific to loss of LytB. We conclude that IPP and DMAPP

production by the last step of the apicoplast DOXP pathway is essential for the *T. gondii* tachyzoite.

3.4.2. DOXPRI, the target of fosmidomycin, is required for parasite growth.

The cyanobacterium *Synechocystis* can, under certain growth conditions, negate the necessity for early steps of the DOXP pathway through metabolite diversion from other pathways(Ershov et al., 2002). This shunt renders IPP synthesis resistant to fosmidomycin treatment. We therefore considered that the final steps of the pathway are required but the early step mediated by DOXPRI is not. To test this, we turned our attention to *T. gondii* DOXPRI directly. Once again, the predicted gene model lacked a targeting peptide. We conducted 5' RACE experiments and, in this case, identified two additional exons 5' of the available model (Fig. 3.10 B). These exons appeared to encode a canonical signal peptide followed by a transit peptide typical of apicoplast proteins (see multiple sequence alignment in Fig. 3.11). The extended coding sequence was engineered into an epitope tagging construct and transformed into parasites. The tagged protein localized to the apicoplast as judged by immunofluorescence staining (Fig. 3.3 A). Using the strategy outlined in the previous section for the LytB gene, we engineered a conditional mutant of the DOXPRI gene (Fig. 3.3 , note that we constructed the mutant using an untagged regulated copy). Successful gene targeting was confirmed by Southern blot analysis (Fig. 3 B and Fig. 3.10 B). We demonstrated tight regulation of the transgene using an antibody raised against recombinant protein expressed in *E. coli*. Next, we studied the impact of this mutation on parasite infection.

We found a pronounced growth defect indistinguishable from the phenotype observed for the LytB mutant. Collectively, these observations strongly suggest that the entire apicoplast DOXP pathway is required for growth and that fosmidomycin resistance in *T. gondii* is not the result of a metabolic shunt that sidesteps the target DOXPRI.

3.4.3. Dependence on a target enzyme with known fosmidomycin sensitivity does not confer sensitivity to the parasite

We next considered that the *T. gondii* DOXPRI enzyme itself may be inherently resistant to fosmidomycin. We expressed various portions of the *T. gondii* DOXPRI gene in *E.coli*. This resulted in robust production of recombinant protein; however, the proteins were largely insoluble and we did not detect significant enzymatic activity. Our inability to directly measure *T. gondii* DOXPRI led us to design an indirect experiment. We wondered if dependence on an enzyme known to be sensitive would render the parasite susceptible. We chose the well characterized DOXPRI from *E. coli*. The IC 50 value of fosmidomycin for this enzyme is 8.2 nM, which is slightly below that of the P. falciparum homologue (Kuzuyama et al., 1998). We introduced the coding sequence of DOXPRI amplified from E. coli into a parasite expression construct that fused it with an apicoplast leader sequence and a Ty-1 epitope tag. As a control, we also engineered the *T. gondii* DOXPRI gene with a Ty-1 tag. These plasmids were introduced into the ΔDOXPRI mutant. As shown in Fig. 3.4 (A and B) , the bacterial protein localizes to the apicoplast and the protein is proteolytically processed as expected. We established by western blot that under ATc the transgenic E. coli protein is the only detectable DOXPRI

enzyme in the Δ DOXPRI+EcDOXPRI parasite strain (Fig. 3.4 B). Next, we scored the impact of ATc and/or fosmidomycin on the growth of this strain in comparison with the mutant and the homologously complemented line. Plaque assays under ATc revealed that the *E. coli* enzyme fully rescues the mutant, as does the parasite enzyme (Fig. 3.4 D). This strongly supports our initial assumption that gene TGGT1_125270 encodes a DOXPRI. Importantly, however, both strains remain resistant to fosmidomycin. This suggests that fosmidomycin resistance in *T. gondii* does not depend on inherent resistance of the target enzyme. We acknowledge that we have not formally established that *T. gondii* DOXPRI is drug sensitive, but we note that even when the parasite depends on a sensitive enzyme it is still drug resistant. Overall, these experiments implicate drug accessibility as the most likely mechanism of resistance. Fosmidomycin is a phosphonic acid (Fig. 3.1 A), a charged compound which does not readily cross membranes by diffusion but requires transport. Cellular membranes therefore represent a barrier to the drug, and numerous membranes separate the target enzyme in the lumen of the apicoplast from the drug in the medium. These include four apicoplast membranes, the parasite plasma membrane, the parasitophorous vacuole (note that this membrane is thought to contain pores permeable to small molecules), and the plasma membrane of the host cell. We performed a series of experiments to establish the relative importance of each of these potential parasite and host barriers.

3.4.4.The host cell is not the critical determinator of parasite fosmidomycin sensitivity

Fosmidiomycin-sensitive apicomplexans parasitize erythrocytes, and the permeability of the host cell membrane may govern fosmidomycin sensitivity (Seeber and Soldati-Favre, 2010). Plasmodium and Babesia massively reorganize the erythrocyte, a process which includes the installation of new permeability pathways in the host cell membrane (Desai et al.,1993 ; Alkhalil et al., 2007). These pathways dramatically change the rate of metabolite uptake and may be required for fosmidomycin transport. This hypothesis makes testable predictions: first, that sensitivity depends on the erythrocyte and it's remodeling in sensitive species; and second, that breaching the host cell permeability barrier will produce sensitivity in resistant species. To explore the first prediction, we conducted experiments with the rodent malaria model Plasmodium berghei during infection of liver cells. Treating parasites at liver stage with 10 µM fosmidomycin produced a distinct cellular phenotype in which overall cellular development appears normal but the development of the apicoplast is blocked (Fig. 3.5 B). This mirrors the response *P. falciparum* parasites show to fosmidomycin treatment in the blood stage (please note that we use *P.falciparum* for comparison in this study because the *P.berghei* erythrocytic cycle cannot be propagated in culture). Again cellular development appears normal except for a loss of apicoplast elongation and division (Fig. 3.5 D). To quantify the effect of fosmidomycin on *P. berghei* parasites in the liver stage, we cultured drug treated and control parasites for 65 h, which normally results in the production of merosomes— groups of merozoites which are packaged in

liver cell membrane and released. Merosomes represent successful completion of the liver stage of the *P. berghei* life cycle. Fosmidomycin treatment signifi cantly and reproducibly reduced the number of merosomes (Fig. 3.5 E), presumably because of the retarded growth of the apicoplast. *Plasmodium* parasites are thus also sensitive to fosmidomycin when developing in nucleated liver cells just as they are in RBCs. Compound penetration into mammalian cells can be enhanced by masking polar groups with a hydrophobic ester (Li et al., 1997). After crossing the membrane, the ester is cleaved by cellular esterases, thus releasing the original compound in the cytoplasm. We have synthesized several such ester derivatives for fosmidomycin (see supplemental material for detail). We tested the effects of varied concentrations of diphenyl-, dimethoxyphenyl-, and dipyvaloxymethyl-fosmdiomycin on the growth of *T. gondii* and note that as fosmidomycin these derivatives are poor parasite inhibitors, showing essentially no activity at the concentrations used (Table 3.1). Fosmidomycin esterification does not provide enhanced efficacy, and this is consistent with the host cell membrane not acting as the main barrier to drug uptake. We note that we do not directly demonstrate enhanced host cell uptake for these compounds.

3.4.5. Expression of a bacterial transporter confers drug sensitivity to *T. gondii*.

We next hypothesized that *T. gondii* may be unable to take up fosmidomycin, or alternatively that the *T. gondii* apicoplast lacks an import mechanism for the drug from the parasite cytoplasm. As the lack of import is difficult to demonstrate in absence of a positive control, we thought to artificially engineer transport and sensitivity. The

sensitivity of a variety of bacterial pathogens to fosmidomycin, and the structurally related antibiotic fosfomycin, depends on the transporter glycerol-3-phosphate (G3P) transporter (GlpT; Sakamoto et al., 2003; Takahata et al., 2010). This transporter facilitates the uptake of sn-G3P in exchange for inorganic phosphate (Law et al., 2009). We amplified the coding sequence for GlpT from E. coli and engineered three parasite expression vectors: the first construct added the full leader of the apicoplast protein FNR at the N terminus of GlpT (L-GlpT), the second used only the N-terminal signal peptide portion (SP-GlpT), and the third did not add any sequences at the N terminus. All these versions carried a Ty-1 epitope. We were able to establish parasite lines expressing the first two transporter versions but did not detect GlpT in the absence of an N-terminal fusion (fusion is known to enhance the expression of heterologous transgenes in *T. gondii* ; Striepen and Soldati, 2007). L-GlpT and the SP-GlpT show a similar immunofluorescence pattern: the protein is present in various parasite membranes including the plasma membrane (Fig. 3.6, A and B). We also detect more intense internal staining close to the nucleus. Colabeling with an anti-Cpn60 antibody shows that this staining does not coincide with the apicoplast (Fig. 3.6 C). We tested the growth of GlpT-expressing parasites in the presence of varied concentrations of fosmidomycin (0.375–100 μM). To facilitate such measurements, we had constructed the GlpT-expressing strains in a fluorescent parasite line. Parasites stably expressing L-GlpT or SP-GlpT were sensitive to fosmidomycin with IC_{50} values of 1.3 μM (LGlpT) and 2.6 μM (Sp-GlpT), respectively (Fig. 3.7, B and C; and see the plaque assay in Fig. 3.12). These values are similar to those previously reported for different *Plasmodium*

and *Babesia* species and at least 100-fold lower than those observed with the RH-YFP-YFP parental strain. Note that because of the strong drug resistance we were unable to establish a precise IC_{50} for wild-type cells. To establish whether fosmidomycin sensitivity in GlpT transgenic parasites is directly dependent on the transport activity of GlpT, we constructed two GlpT mutants. G3P is thought to bind to two critical arginine residues at positions 45 and 269 that are exposed during substrate loading (Huang et al., 2003). Upon G3P binding, the protein changes conformation in a rocker switch–type movement that results in translocation of the substrate. Mutations of these residues affect binding and transport of G3P (Law et al., 2008). We engineered an R45K and an R45AR269K double replacement into L-GlpT by site-directed mutagenesis that were introduced into YFP-expressing parasites. Mutant proteins were expressed with localization and expression level equivalent to the L-GlpT strain as judged by immunofl uorescence and Western blot (Fig. 3.7 F and Fig. 3.13). Parasites expressing the single mutant showed a fourfold drop in fosmidomycin sensitivity when compared with L-GlpT parasites (IC 50 = 5.7 μM), whereas those carrying the double mutant were fully resistant to fosmidomycin (Fig. 3.7, D and E). We conclude that expression of the bacterial GlpT confers a dramatic increase in fosmidomycin sensitivity. Sensitivity depends on transporter activity but appears not to require apicoplast localization of the protein. We next tested esterified fosmidomycin analogues against the L-GlpT line and again established IC_{50} values (Table 3.1). The IC_{50} values for diphenyl- and dimethoxyphenyl- fosmidomycin were indinstinguishable from those for underivatized fosmidomycin (1 and 2 μM, respectively). Dipyvaloxymethyl-fosmdiomycin shows poor

efficacy in wild type and transgenics. Esterification thus did not produce enhancement beyond the dramatic effect of GlpT expression. Collectively, our results indicate that the parasite, and not the host, represents the main barrier to fosmidomycin. To ensure that our conclusion is not limited to our tissue-cultured human fibroblasts, we conducted in vivo experiments (Fig. 3.7G). Webster mice were infected with 20 L-GlpT expressing *T. gondii* by intraperitoneal injection in two groups of five. One group of mice was treated with a daily injection of 100 mg/kg fosmidomycin in PBS for 10 consecutive days, and a second group was mock treated with PBS alone. The GlpT transgenic parasite line is derived from the highly virulent type I strain RH, and untreated mice died after 10 d. Treated mice did not show any signs of disease for the observation period of 30 d. Western blot analysis of sera drawn from treated mice indicated that mice were successfully infected. The trial was repeated with three mice in each group with similar outcome (note that the amount of available drug limited the group size). We conclude that fosmidomycin treatment for 10 d cures mice from a lethal challenge infection of GlpT expressing RH strain parasites.

3.4.6. GlpT expression enables uptake of fosmidomycin in transgenic parasites.

We next asked whether fosmidomycin sensitivity in GlpT expressing parasites is a result of increased drug uptake. To test this, we used growth inhibition of *E. coli* as a simple but sensitive bioassay for fosmidomycin. By exposing the bacteria to parasite lysate in a classical agar plate diffusion assay, we would determine if parasites had taken up fosmidomycin. Wild-type parasites and our transgenic lines expressing GlpT

were exposed to fosmidomycin, and parasites were harvested by centrifugation, washed, and disrupted by sonication. The parasite lysate was loaded onto paper discs and these were layered on agar plates inoculated with *E. coli*. Bacteria were then allowed to grow for 24 h. Fig. 3.8 A shows discs impregnated with known amounts of fosmidomycin. The bacteriocidal activity of the drug is visible as clear zones of inhibition against an opaque bacterial lawn. The diameter of these zones is proportional to the amount of drug, and we established a standard curve from 1 to 200 ng. We next compared wildtype parasites (RH) with the GlpT transgenic lines (Fig. 3.8 B). Although we detect no fosmidomycin in RH wild type (<1ng/10 7 cells), we note robust inhibition zones in the GlpT expressing parasites equivalent to 28 and 23.4 ng for L-GlpT and Sp-GlpT, respectively. Intracellular fosmidomycin concentrations were reduced in parasites expressing the transporter with the single R45K mutation (10.4 ng) and were equivalent to untransfected parasites in those expressing the R45K-R269K double-mutant transporters (Fig. 3.8 D). We conclude that wild-type *T. gondii* are incapable of accumulating fosmidomycin and that expression of GlpT enables robust drug uptake. Permeabilizing the parasite plasma membrane removes a significant barrier for fosmidomycin. We conducted additional experiments to biochemically define the parasite barrier to fosmidomycin flux and its breach by GlpT. Work on organellar tRNAs of *T. gondii* has demonstrated dramatic differences in the detergent sensitivity of the plasma membrane and the membranes of the apicoplast (Esseivaet al., 2004). Cytoplasmic tRNAs were susceptible to RNase digestion after treatment with 0.1% digitonin, whereas apicoplast tRNAs remained protected. Although it is technically

challenging to measure uptake in the presence of detergent, measuring release from parasites is feasible using the assay we developed. We incubated wild type and L-GlpT–expressing parasites with fosmidomycin. Next, we exposed parasites to 0.1% digitonin for 5 min on ice followed by centrifugation to separate the detergent extract from the cell pellet, which includes the apicoplast. Residual cells in the pellet were then broken as described in the previous section, and we measured the fosmidomycin concentration in both fractions using the bioassay. As shown in Fig. 3.8 (E and F), digitonin treatment released 17.6 ng, or ~86% of the total parasite-associated fosmidomycin from the L-GlpT parasites. Note that digitonin treatment in the absence of fosmidomycin does not produce an inhibition zone. To control for the membrane specificity of digitonin, we evaluated a parasite line that expresses YFP in the cytoplasm and RFP in the apicoplast by fluorescence microscopy and flow cytometry. Fig. 3.8 (G and H) shows that digitonin-treated parasites loose cyptoplasmic YFP while maintaining red fluorescence in the plastid. We note that the majority of fosmidomycin can be released by specific permeabilization of the parasite plasma membrane.

3.5. DISCUSSION

The apicoplast has several attractive attributes for drug development. It is home to literally hundreds of proteins, many of which are enzymes, and many of these enzymes can be inhibited using drug-like small molecules (Wiesner et al., 2008). There are established targets among the enzymes that replicate, transcribe, and translate the

apicoplast genome (Dahl and Rosenthal, 2008), and some of these (azithromycin or tetracycline) are already in clinical use for toxoplasmosis or malaria.

3.5.1. The DOXP pathway is a good target for the development of antiparasitic drugs.

A second important group of potential targets is enzymes central to apicoplast anabolism, in particular the FASII and DOXP pathways for the synthesis of fatty acids and isoprenoid precursors, respectively. These enzymes are of cyanobacterial origin, and their pronounced evolutionary and mechanistic divergence from those used by the human host offers the potential of specificity. Significant efforts have been made to characterize the enzymology of both pathways and to discover, and iteratively optimize, inhibitors. As discussed in more detail in the introduction, the example of the FASII pathway argues for the rigorous analysis not only of the diversity and drugability of the chosen target but also of its essentiality. Essentiality is a complex phenomenon, and, surprisingly, pronounced differences are found among apicomplexa depending on the parasite species, the life cyclestage, and the host cell niche occupied by that stage. There is pharmacological support for a critical role of the DOXP pathway for *Plasmodium* and *Babesia* , yet many other apicomplexa are resistant to fosmidomycin. In this study, we provide evidence that a functional DOXP pathway is an absolute requirement for the growth and survival of *T. gondii* despite the parasite's resistance to fosmidomycin. We engineered conditional null mutants for an early and a late enzymatic step in the pathway, and both mutants are lethal. We note in this paper that growth

ceases very rapidly upon downregulation of these conditional alleles. As shown in Fig. 3.14 ,the kinetics of inhibition are faster than those of loss of FASII and similar to those obtained by ablation of the APT (Brooks et al., 2010). Our experiments argue that the DOXP pathway represents a valid target across the phylum. They also support the notion of isoprenoid precursor synthesis as a key function of the apicoplast. Although it is now clear that IPP production in the apicoplast is essential, it is less clear why. Numerous potential functions of IPP-derived molecules have been discussed. They may be apicoplast specific (ispoprenylation of tRNAs), act in other organelles (providing dolichol or ubiquinone to the parasite ER or the mitochondrion, respectively), or more broadly control parasite behavior and development (abscisic acid and other isoprenoid plant hormones; Moreno and Li, 2008). The mutants established in this study provide an excellent starting point to uncover the most critical metabolite and its function for the parasite. Uncovering this function may lead to additional targets.

3.5.2. Fosmidomycin uptake governs parasite sensitivity.

In the current study, we have tested a series of mechanisms potentially underlying fosmidomycin resistance in *T. gondii* . We did not find direct evidence for redundancy as a result of product salvage, metabolic shunting of the target, or inherent drug resistance of the target enzyme. A recent study in *P. falciparum* has shown that fosmidomycin resistance induced by invitro selection was associated with copy number variation of the gene encoding the target enzyme (Dharia et al., 2009). It is conceivable that *T. gondii* also overexpresses DOXPRI and that this confers resistance, but we

deem this unlikely. Overexpression in *Plasmodium* conferred a relatively modest eightfold increase in the IC_{50}, whereas the difference in sensitivity between Plasmodium and *T. gondii* is several 100-fold. Overall, our data are most consistent with a lack of drug access as the primary mechanism of insensitivity. The key observations supporting this hypothesis are: (1) that we fail to detect drug uptake in wild-type tachyzoites (Fig. 3.8), and (2) that we can render parasites drug sensitive by engineering uptake using a fosmidomycin transporter. Expression of this transporter reduces the IC_{50} >100-fold to 1.5 μM, a value comparable to sensitive species. Furthermore, we demonstrate that these transgenic parasites now accumulate fosmidomycin and that point mutations that attenuate transport also attenuate drug efficacy. Lack of uptake has also been described as a major mechanism for fosmidomycin resistance in bacteria. This includes natural resistance as documented for Mycobacterium (Dhiman et al., 2005; Brown and Parish, 2008) as well as acquired resistance in response to drug pressure (Sakamoto et al., 2003; Takahata et al., 2010). Loss of drug import is therefore a potential threat to the clinical use of fosmidomycin as an antimalarial. That loss of the transporter was not readily observed in resistant malaria parasite (Dharia et al., 2009) may indicate that the transporter is essential and therefore not as easily lost as it is in bacteria. Understanding the true metabolic function of the transporter will help to gauge the probability of its loss and the development of resistance. Alternatively, identifying DOXP pathway inhibitors that do not require a specific transporter to reach the target could circumvent this issue. Such inhibitors should also show broader efficacy against other apicomplexans. Most of the DOXP pathway intermediates are highly charged

compounds, and thus many of the inhibitors identified so far are charged as well. However, there are exceptions, like the herbicide ketoclomazone, an inhibitor of DOXP synthase which has recently been found to have antibacterial activity (Matsue et al., 2010).

3.5.3. What governs fosmidomycin uptake?

Why do only some apicomplexan parasites take up fosmidomycin? Our results support a model in which the parasite plasma membrane of *Plasmodium* has fosmidomycin transport activity that appears to be absent or diminished in *T. gondii* . This hypothetical transporter likely imports metabolites from the host cell, and fosmidomycin hitches a ride as an uninvited and deadly passenger. As demonstrated by GlpT, G3P is a strong (but likely not the only) potential candidate for such a metabolite. In *E. coli* , imported G3P serves as a major source of carbon and energy but also supplies glycerophospholipid synthesis (Huang et al., 2003). The demand for glycerophospholipids of *Plasmodium* in the RBC is very heavy and met by a complex web of pathways using elements of salvage and de novo synthesis (Déchamps et al., 2010). Plasmodium can synthesize G3P through a glycerol kinase, for example; however this enzyme is poorly expressed and not essential during RBC development, arguing for additional sources (Schnick et al.,2009). The next downstream step is the acylation of G3P. Interestingly, both *Plasmodium* and *T. gondii* encode two enzymes for the initial sn-1 reaction, one localized to the ER and one to the apicoplast (Déchamps et al., 2010). Consumption of G3P in the apicoplast could require G3P import, and the

yet-to-be-identified plastid GlpT may be the route that fosmidomycin takes to its target in the apicoplast lumen. Our uptake studies (Fig. 3.8) argue that in *T. gondii* the plasma membrane, and most likely not the apicoplast membrane, is the impediment for fosmidomycin. This would fit the shared distribution of G3P acyl transferases. The activity of G3P dehydrogenase provides an alternative route to G3P from dihydroxyacetone-phosphate. Both *Plasmodium* and *T. gondii* encode two genes and, at least in the case of *Plasmodium* , one of the proteins carries a signal peptide that could hint apicoplast localization (Ralph et al., 2004). In that case, there may not be need for G3P import into the apicoplast, as triose phosphates are plentiful as a result of the activity of the APT. Detailed biochemical experiments are needed to penetrate the complexity of these metabolic pathways. Metabolomic studies tracing stable isotope labeled G3P in different apicomplexa could offer a way to further evaluate the model. In summary, our analysis of fosmidomycin sensitivity in *T. gondii* and *Plasmodium* has validated the target DOXPRI, has pointed out potential mechanisms of resistance, and has generated testable hypotheses and reagents to further dissect the metabolic basis of apicoplast function and drug sensitivity

Figure 3.1: *Toxoplasma* is resistant to fosmidomycin (A) Schematic representation of apicoplast fatty acid and isoprene biosynthesis pathways (highlighted by white boxes). Both pathways depend on substrates imported from the cytoplasm by the apicoplast phosphate translocator (APT). Note the structural similarity between DOXP and fosmidomycin. Selected enzymes are italicized: PDH, pyruvate dehydrogenase; DOXPS, DOXP synthase; DOXPRI, DOXP reductoisomerase; ACP acyl carrier protein. DOXP pathway intermediates: MEP, methylerythritol-phosphate; CDP-ME, cytosine dinucleotide phosphate methylerythritol; MEcPP, methylerythritol cyclopyrophosphate; HMBPP, hydroxymethy-but-enyl pyrophosphate. Apicoplast is shown bounded by a single membrane for simplicity. **(B)** Growth of *T. gondii* in the presence or absence of fosmidomycin (Fos). Fluorescence data are shown as percent of the highest value of the untreated control. Data points reflect the mean of duplicate wells and error bars show standard deviation, where no bar is shown it was smaller than the symbol. All following fluorescence assays are shown in this way

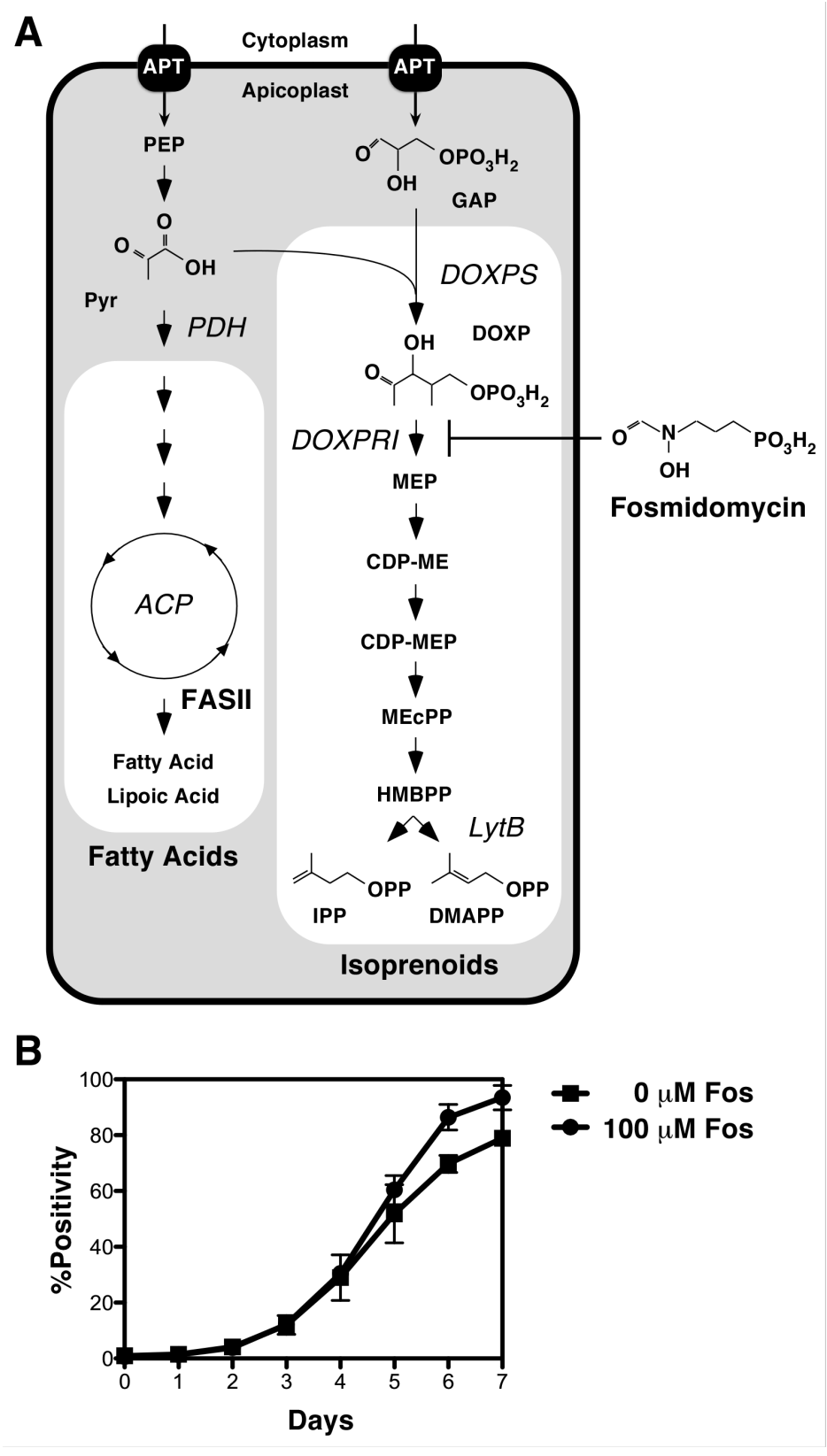

A
Cytoplasm
APT
APT
Apicoplast
PEP
GAP
Pyr
PDH
DOXPS
DOXP
DOXPRI
MEP
CDP-ME
CDP-MEP
MEcPP
HMBPP
LytB
IPP
DMAPP
Fosmidomycin
ACP
FASII
Fatty Acid
Lipoic Acid
Fatty Acids
Isoprenoids
B
%Positivity
Days
0 μM Fos
100 μM Fos

Figure 3.2: LytB, the terminal step of the DoxP pathway, is indispensible (A) Immunofluorescence assay of parasites expressing epitope tagged LytB. Cells were counterstained for the apicoplast protein ACP and DNA (DAPI). (**B**) Southern blot analysis of BglII restricted genomic DNA of wild type (RH), LytB parental (iLytB) and conditional mutant (ΔLytB) probed with a PCR product representing the first exon and intron. The native locus is predicted to produce two bands (2.9 kb and 3.2 kb see Fig. S2A for a map), the ectopic regulated copy a single 8 kb fragment. (**C**) Western blot of protein extracts of parasites cultured for 0-3 days under ATc. pLytB, LytB precursor; mLytB, mature LytB; both detected using an antibody to the myc epitope tag. Dense granule protein 8 (GRA8) serves as loading control. **(D)** Plaque assays comparing the growth of the LytB mutant (ΔLytB) with the parental line (iLytB) and a mutant clone expressing a complementing copy of the gene under a constitutive promoter (ΔLytB Comp). **(E)** Fluorescence growth assays for the same parasite lines growing in the presence (+ATc) or absence of ATc (-ATc), or after preincubation with ATc (3d +ATc).

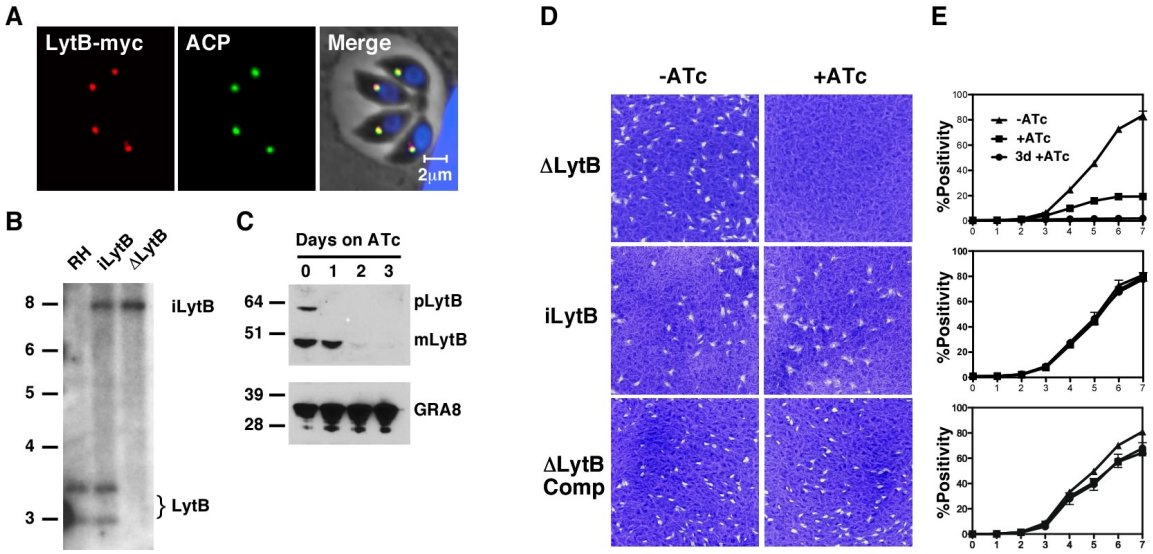

A
LytB-myc
ACP
Merge
2μm
B
RH
iLytB
ΔLytB
8
6
5
4
3
iLytB
LytB
C
Days on ATc
0 1 2 3
64
51
pLytB
mLytB
39
28
GRA8
D
-ATc
+ATc
ΔLytB
iLytB
ΔLytB Comp
E
%Positivity
-ATc
+ATc
3d +ATc

Figure 3.3: DOXPRI, the target of fosmidomycin, is required for parasite growth

(A) Immunofluorescence of a parasite clone expressing a myc-tagged DOXPRI under the control of the tubulin promoter. (**B**) Southern blot using the coding sequence of DOXPRI as probe comparing wild type (RH), parental (IDOXPRI) and conditional mutant (DDOXPRI). Xho1 restriction of the native locus produces a fragment of 3.8 kb (see Fig. S2B for a map), the regulatable ectopic locus produces a 0.9 kb fragment. (**C**) Western blot measuring the amount of DOXPRI in the mutant upon ATc treatment using an antibody raised against recombinant *T. gondii* DOXPRI. **(D)** Fluorescence or (**E**) plaque assay measuring the growth of mutant and parental strain in the presence or absence of ATc (see Fig. 2E for detail).

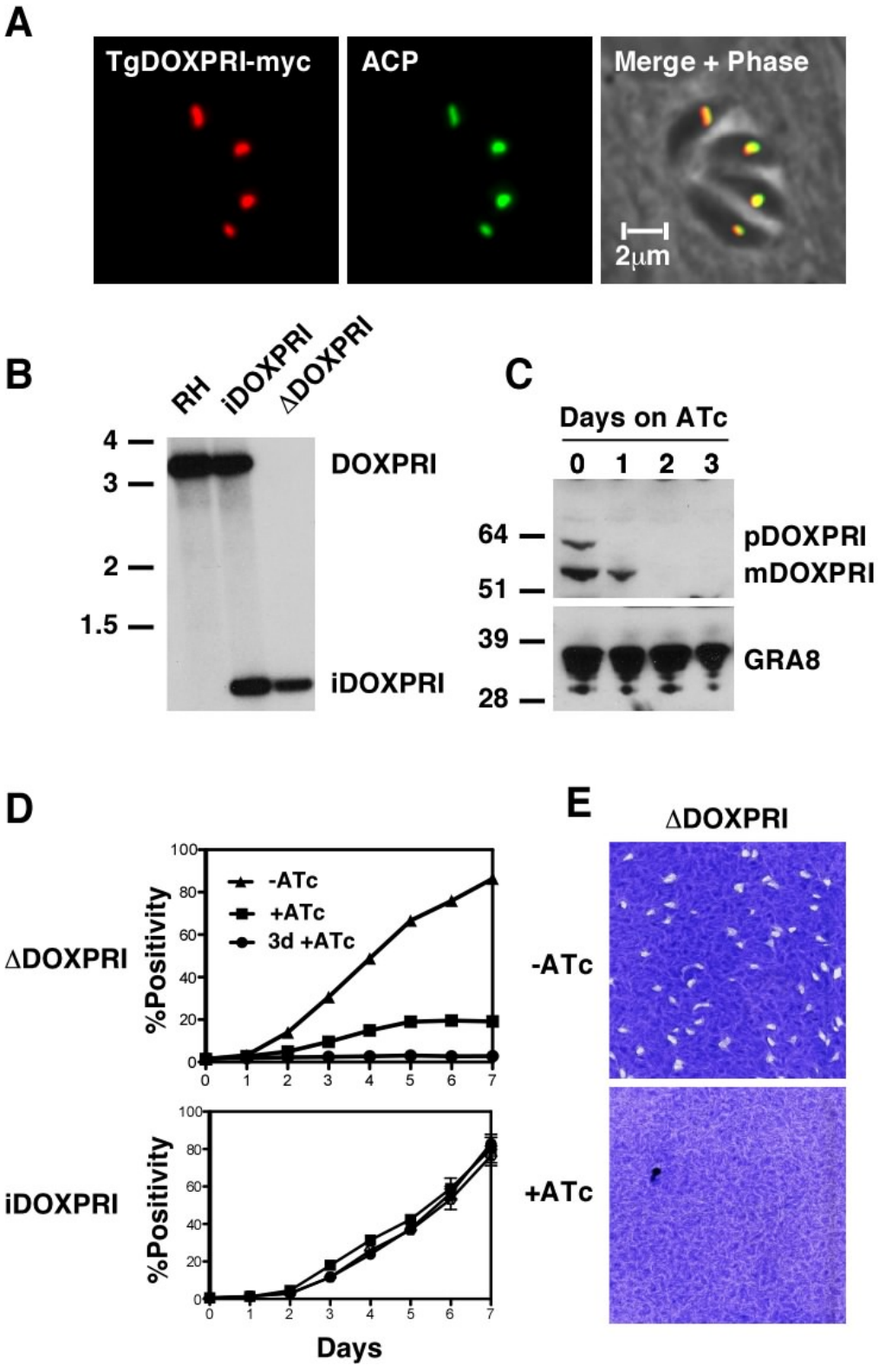

A
TgDOXPRI-myc
ACP
Merge + Phase
2μm
B
RH
iDOXPRI
ΔDOXPRI
4
3
2
1.5
DOXPRI
iDOXPRI
C
Days on ATc
0 1 2 3
64
51
39
28
pDOXPRI
mDOXPRI
GRA8
D
ΔDOXPRI
-ATc
+ATc
3d +ATc
%Positivity
iDOXPRI
Days
E
ΔDOXPRI
-ATc
+ATc

Figure 3.4: Reliance of *E. coli* DOXPRI does not render *Toxoplasma* sensitive to fosmidomycin (A) Immunofluorescence assay showing the localization of Ty-1 tagged *E. coli* DOXPRI fused to an apicoplast leader (green, L-EcDOXPRI). The apicoplast protein Cpn60 is shown for comparison (blue). Western blot analysis of the DDOXPRI mutant complemented with L-EcDOXPRI (**B**) or an ectopic copy of the coding sequence of the *T. gondii* enzyme under the control of a constitutive promoter (TgDOXPRI, **C**). In (**B**) blots were probed with the Ty-1 antibody or the *T. gondii* DOXPRI antibody. Note that under ATc only the *E. coli* enzyme is detectable. (**D**) Plaque assays of the conditional DOXPRI mutant without complementation (top), complemented with the *E. coli* enzyme (middle), or complemented with the *Toxoplasma* enzyme (bottom). Cultures were grown for 10 days in the absence or presence of 0.5 mg/ml ATc and 100 mM fosmidomycin as indicated.

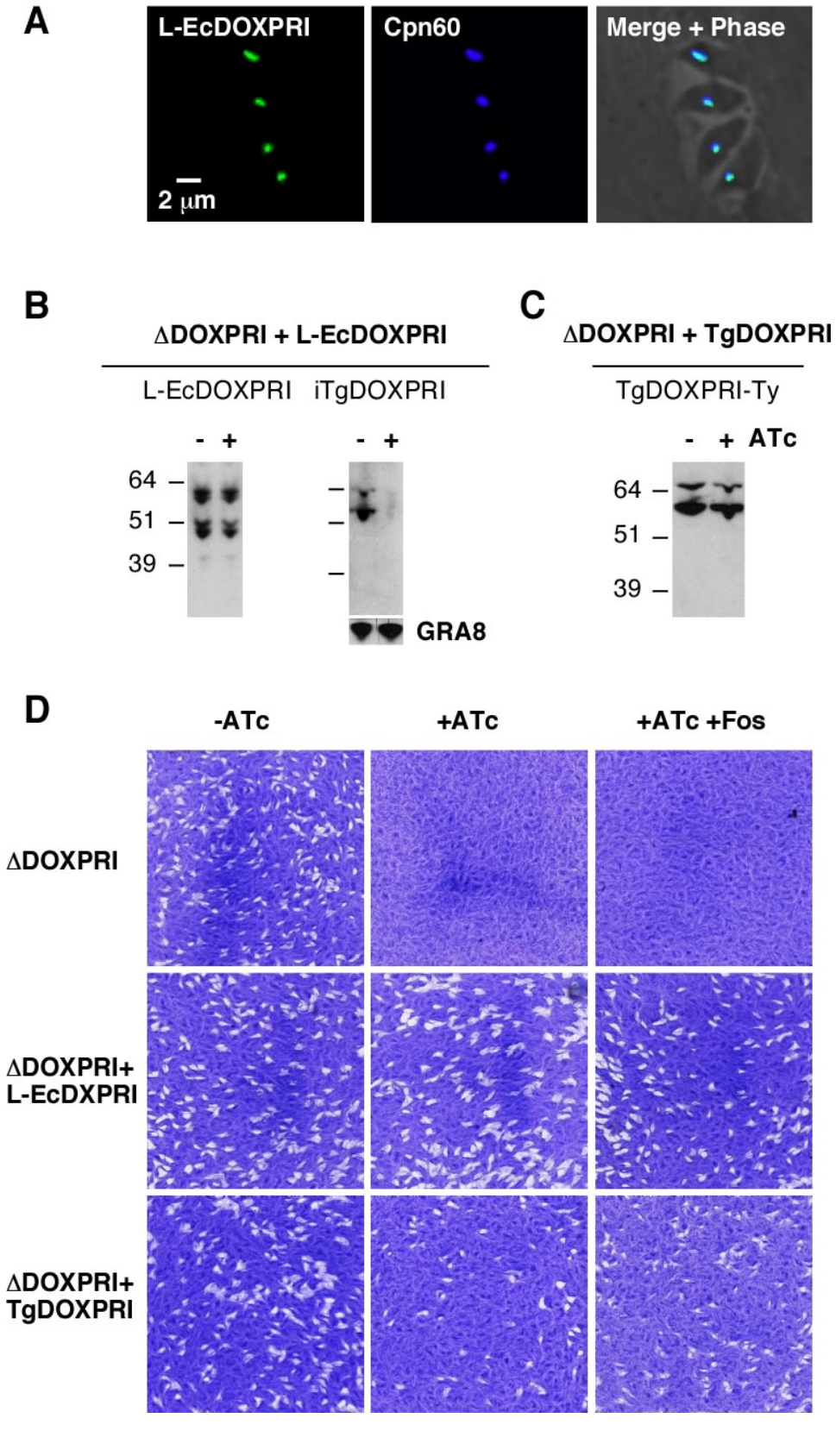
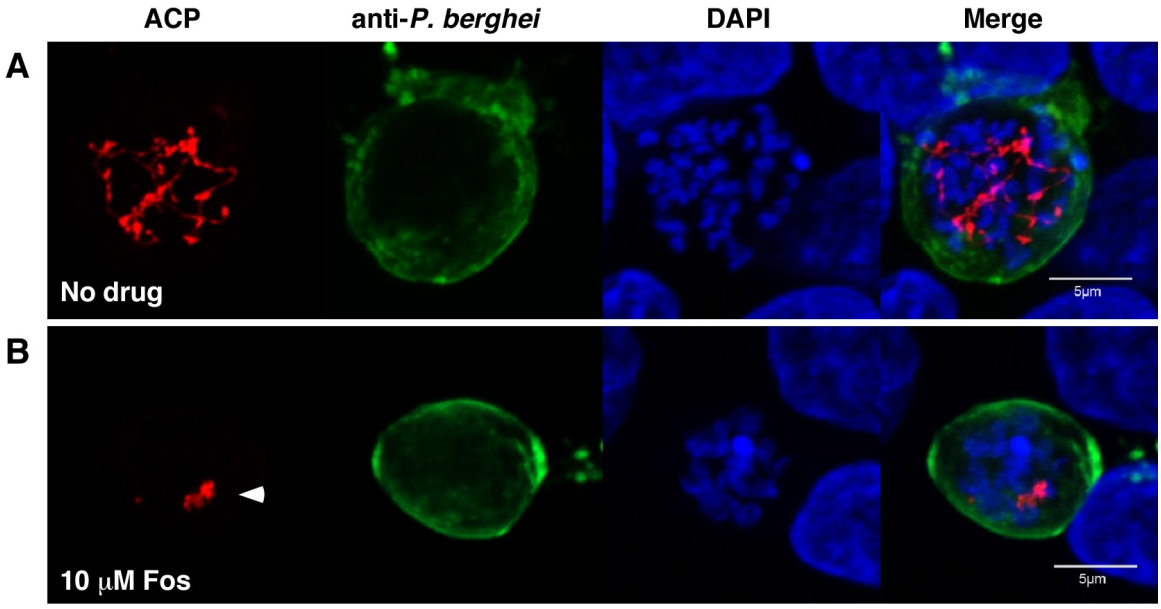
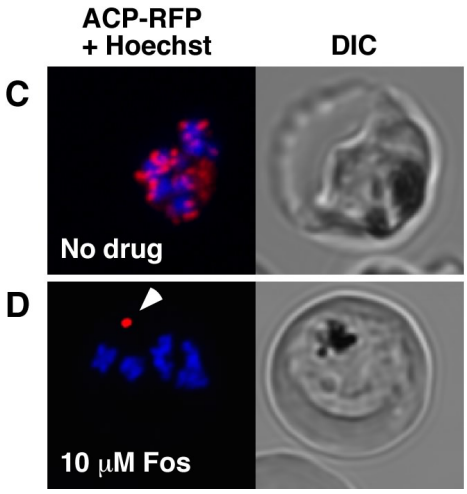

A
L-EcDOXPRI
Cpn60
Merge + Phase
2 μm
B
ΔDOXPRI + L-EcDOXPRI
L-EcDOXPRI
iTgDOXPRI
- +
- +
64
51
39
GRA8
C
ΔDOXPRI + TgDOXPRI
TgDOXPRI-Ty
- + ATc
64
51
39
D
-ATc
+ATc
+ATc +Fos
ΔDOXPRI
ΔDOXPRI+
L-EcDXPRI
ΔDOXPRI+
TgDOXPRI

Figure 3.5: Fosmidomycin affects *Plasmodium berghei* apicoplast liver cell development and merosome formation. (**A**, **B**) Immunofluorescence assay of *P. berghei* infected liver cells. Apicoplast (red) labeled with anti-ACP antibody, parasite (green) labeled with antibody raised to whole *P. berghei* parasites, DNA labeled with DAPI. Underdeveloped apicoplast highlighted by white arrowhead. Note that this phenotype mirrors the effect of other drugs targeting the apicoplast in liver stages (Friesen et al., 2010). (**C**, **D**) Live cell images of *P. falciparum* infected erythrocytes. Cells shown harbor parasite schizonts expressing RFP localized to the apicoplast (red) and nuclei are stained with Hoechst 33342 (blue). Underdeveloped apicoplast indicated by white arrow. (**E**) *P. berghei* liver stage development as measured by the production of merosomes. Treatment with10 μM fosmidomycin reduces successful completion of liver stage infection to 55.8% of untreated control. Merosome counts were conducted on pairwise samples. Treatment and control well were infected from a pooled sporozoite preparation to ensure uniformity. Note that absolute merosome numbers vary between biological replicates (79-458 for the untreated control), however, the relative level of reduction due to fosmidomycin treatment is highly consistent (n=9 independent experiments; paired T-test = $p< 0.0001$)


ACP
anti-P. berghei
DAPI
Merge
A
No drug
5μm
B
10 μM Fos
5μm


ACP-RFP
+ Hoechst
DIC
C
No drug
D
10 μM Fos

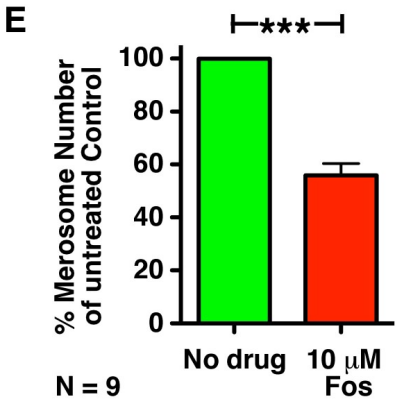

E
***
% Merosome Number of untreated Control
100
80
60
40
20
0
No drug
10 μM Fos
N = 9

Figure 3.6: Expression of the *E. coli* glycerol-3-phosphate transporter.

Immunofluorescence staining of Ty-1 tagged *E. coli* GlpT (red) in parasites expressing the transporter fused downstream of the (**A** and **C**) full apicoplast leader peptide of FNR (L-GlpT) or (**B**) the signal peptide portion of the leader (SP-GlpT). These strains were constructed in a YFP expressing parasite and YFP and DAPI are shown to indicate nucleus and cytoplasm, respectively. Note that the internal staining is distinct from the apicoplast labeled with an antibody to Cpn60 (blue, **C**). Insets in (**C**) show the apicoplast at higher magnification (200%).

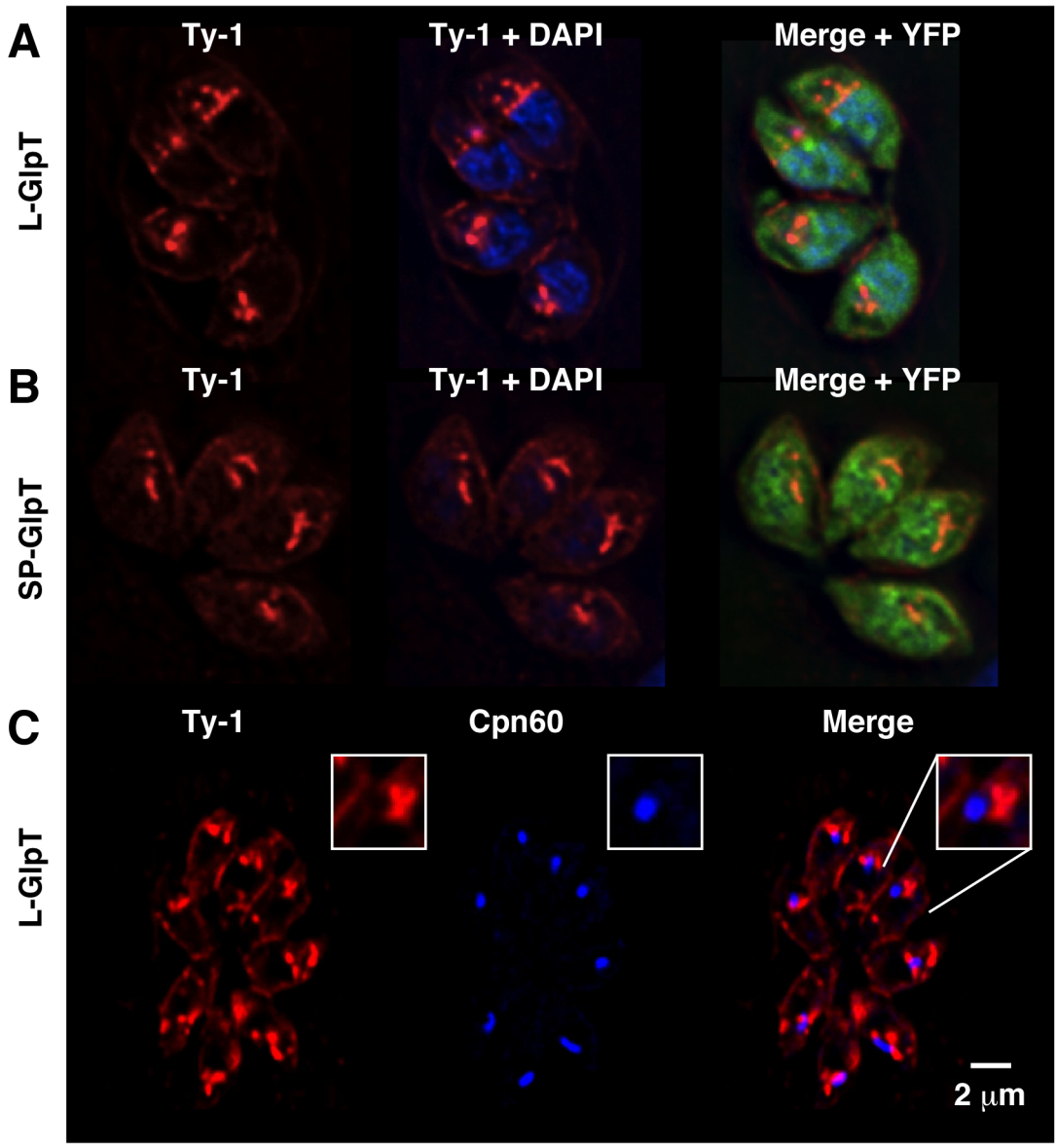

A
Ty-1
Ty-1 + DAPI
Merge + YFP
L-GlpT
B
Ty-1
Ty-1 + DAPI
Merge + YFP
SP-GlpT
C
Ty-1
Cpn60
Merge
L-GlpT
2 μm

Figure 3.7: The glycerol-3-phosphate transporter confers fosmidomycin sensitivity to *Toxoplasma*.

Fluorescence assays measuring the growth of RH-YFP-YFP parasite (**A**) and transgenic clones derived from this line expressing L-GlpT **(B),** SP-GlpT **(C)**, and two point mutants of L-GlpT R45K (**D**) or R45K-R269K (**E**). Assays were conducted using a serial dilution series of fosmidomycin from 100 to 0.375 mM and IC_{50} values were calculated based on growth at day 4. Only three drug concentrations are shown here for simplicity: 0 (squares), 3.1 (triangles) and 100 mM (circles). (**F**) Western blot analysis using the anti Ty-1 antibody indicates a comparable level of GlpT expression in all lines, a-tubulin serves as loading control. (**G**) Mice were infected with 20 L-GlpT parasites each. Infected mice were treated once daily for 10 days (open symbols). Control mice were mock treated (closed symbols) and percent survival is shown for both groups. The trial was conducted with 5 mice in each group (circles) and repeated independently with 3 mice per group (squares). All untreated mice were dead by day 13, treated mice showed no sign of disease.

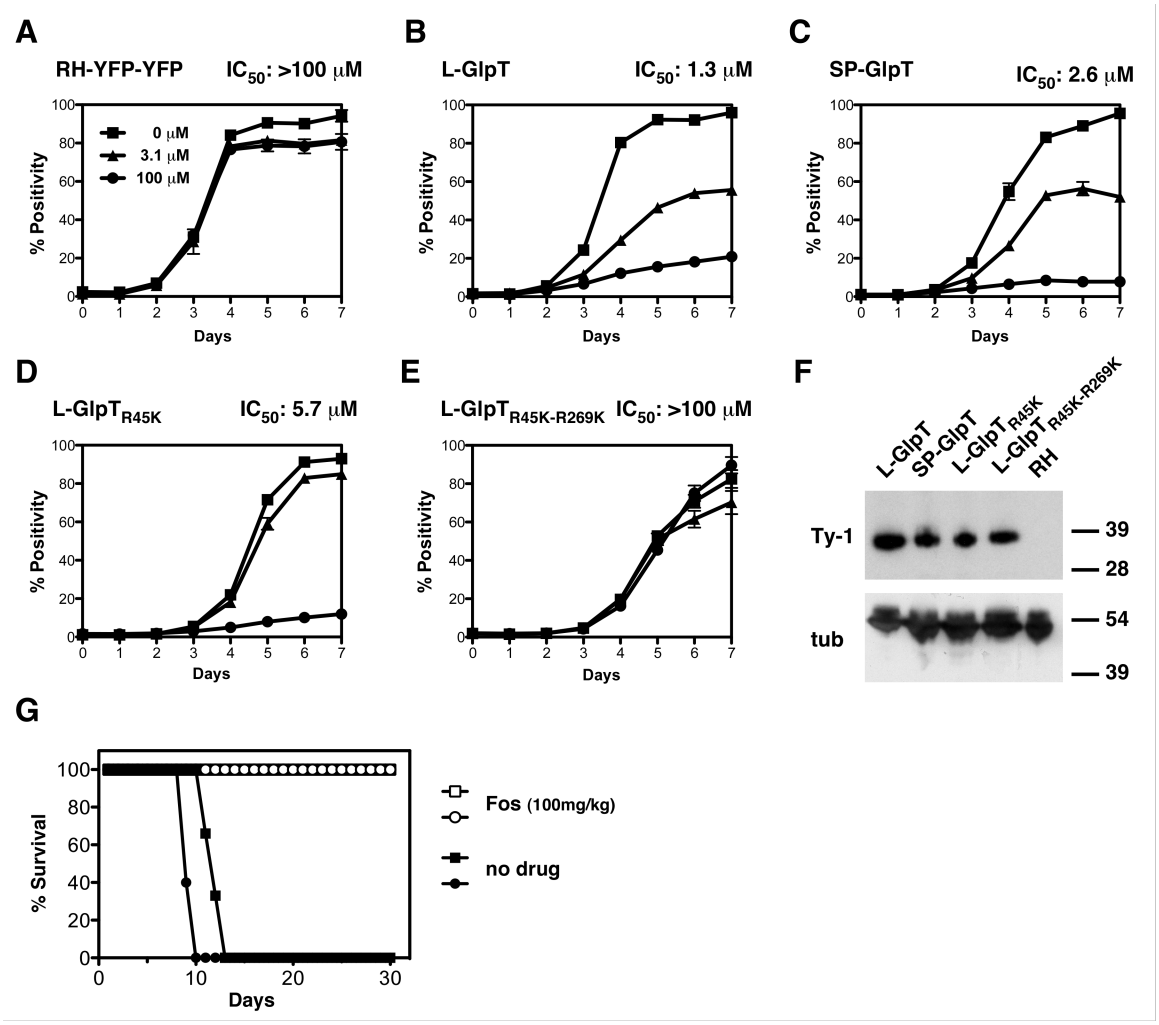

A
RH-YFP-YFP
IC50: >100 μM
0 μM
3.1 μM
100 μM
% Positivity
Days
B
L-GlpT
IC50: 1.3 μM
C
SP-GlpT
IC50: 2.6 μM
D
L-GlpTR45K
IC50: 5.7 μM
E
L-GlpTR45K-R269K
IC50: >100 μM
F
L-GlpT
SP-GlpT
L-GlpTR45K
L-GlpTR45K-R269K
RH
Ty-1
tub
39
28
54
39
G
% Survival
Fos (100mg/kg)
no drug

Figure 3.8: GlpT expression enables fosmidomycin accumulation in transgenic parasites. (A) Bioassay to measure the amount of fosmidomycin using *E. coli*. Note clear inhibition zones against opaque bacterial lawn. Diameter of inhibition zones for a series of known amounts of fosmidomycin was used to establish a standard curve (**C**). (**B**) The indicated parasite strains were exposed to 100 mM fosmidomycin and the amount of fosmidomycin taken up by each strain was measured by bioassay. (**D**) Inhibition zones were measured and transformed into drug amounts using the standard curve shown in (**C**). Note robust fosmidomycin uptake in GlpT expressing parasite absent in RH and the R45K-R269K mutant. (**E**) Parasites were exposed to fosmidomycin as in (**B**) and treated with 0.1% digitonin prior to centrifugation into extract and cell pellet. Fosmidomycin was measured in both fractions by bioassay and (**F**) shows a quantitative analysis of these experiments (data points and bars in **C**, **D** and **F** show the means of duplicate experiments and standard deviation as error bar). A parasite strain that expresses YFP-YFP in the cytoplasm (green) and RFP in the apicoplast (red) was used to assess the specificity of the digitonin treatment by live cell fluorescence microscopy (**G**) and flow cytometry (**H**). Note that the small difference in the red channel between red only and red and green parasites is due to compensation.

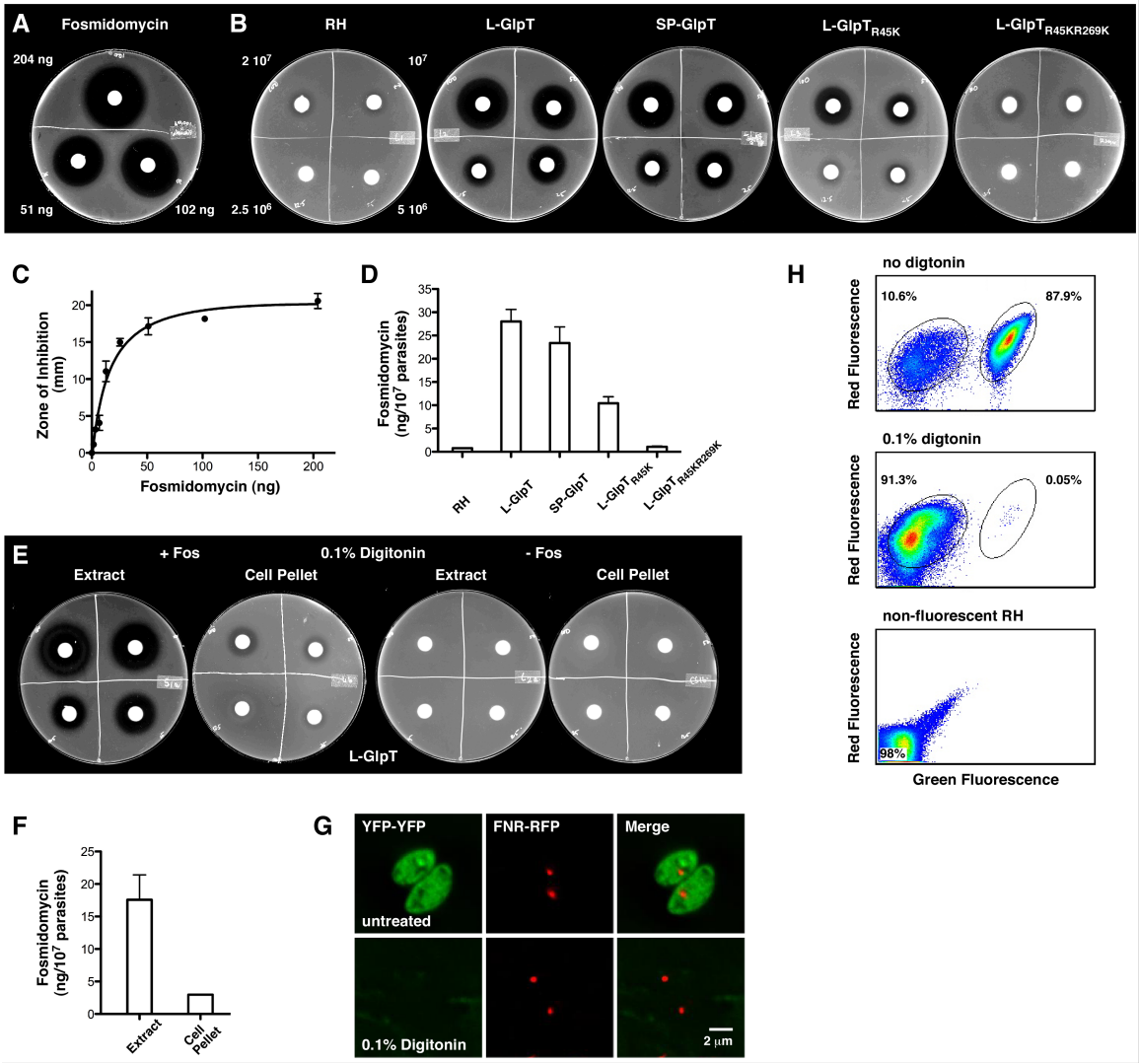
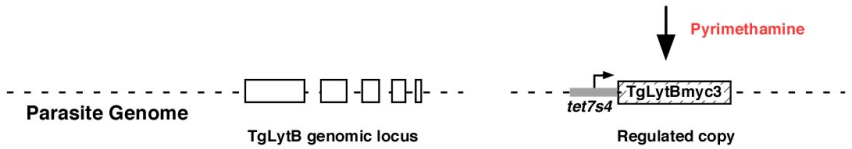
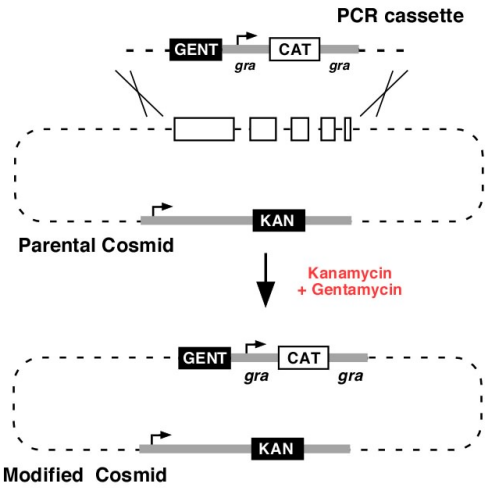

A
Fosmidomycin
204 ng
51 ng
102 ng
B
RH
L-GlpT
SP-GlpT
L-GlpT$_{R45K}$
L-GlpT$_{R45KR269K}$
2 10^7
10^7
2.5 10^6
5 10^6
C
Zone of Inhibition (mm)
Fosmidomycin (ng)
D
Fosmidomycin (ng/10^7 parasites)
RH
L-GlpT
SP-GlpT
L-GlpT$_{R45K}$
L-GlpT$_{R45KR269K}$
E
+ Fos
0.1% Digitonin
- Fos
Extract
Cell Pellet
Extract
Cell Pellet
L-GlpT
F
Fosmidomycin (ng/10^7 parasites)
Extract
Cell Pellet
G
YFP-YFP
FNR-RFP
Merge
untreated
0.1% Digitonin
2 µm
H
no digtonin
10.6%
87.9%
Red Fluorescence
0.1% digtonin
91.3%
0.05%
Red Fluorescence
non-fluorescent RH
Red Fluorescence
98%
Green Fluorescence

Figure 3.9: Schematic outline of the genetic manipulations used to construct conditional mutants in this study. The manipulation of the LytB locus is shown as an example. The drugs used to select for stable transformants at each step are highlighted in red (note that kanamycin and gentamycin are used to select for a recombined targeting cosmid in bacteria, all others for selection in parasites). Markers used: GENT, gentamycin resistance marker (Brooks et al., 2010; Poteete et al., 2006); CAT, chloramphenicol acetyl transferase (Kim et al., 1993; Soldati and Boothroyd, 1993); KAN, kanamycin resistance marker (Gubbels et al., 2008); DHFR-TS, pyrimethamine resistance marker in 1 ((Donald and Roos, 1993), marker not shown); BLE, phleomycin marker used in 4 ((Soldati et al., 1995), marker not shown). Promoter and 5' UTR sequences used: tet7s4, a tetracycline regulated promoter element constructed from the core promoter of the SAG4 gene and 7 tet operator sites (Meissner et al., 2002); gra, promoter and UTR sequences from the GRA1 gene (Cesbron-Delauw et al., 1989); tub, promoter and 5' UTR sequences from the a-tublin gene ((Nagel and Boothroyd, 1988), 3' UTR in this and the regulated expression plasmid are derived from the DHFR-TS gene). See (Brooks et al., 2010) for further detail on recombineering of cosmid clones for genetic engineering in *T. gondii* and (Striepen and Soldati, 2007) for further detail and reference on specific promoters and selectable markers

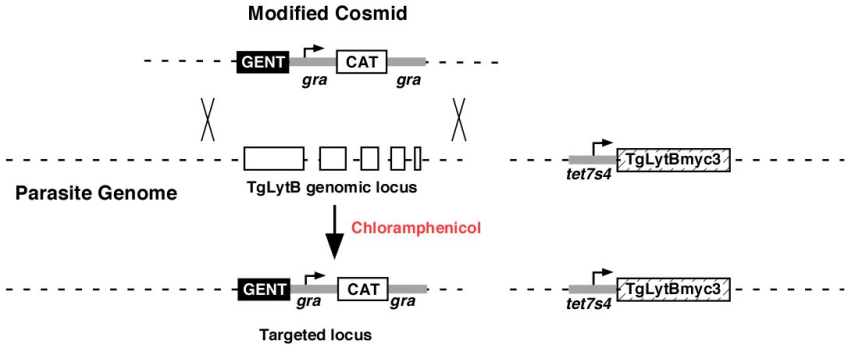
1. Introduction of ectopic copy under tet-regulatable promoter



2. Recombineering of gene-targeting cosmid



3. Deletion of the endogenous locus



4. Functional complementation using a minigene under a constitutive promoter

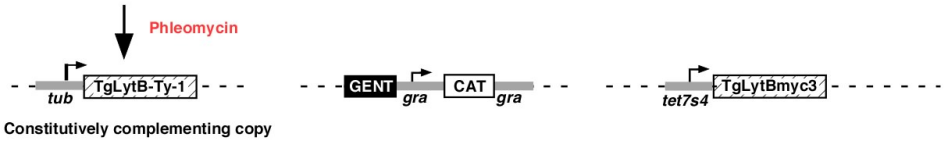


Figure 3.10: Locus restriction map for the *T. gondii* LytB and DOXPRI genes. The restriction sites of the enzymes used in Southern analysis for (**A**) LytB (BglII) and (**B**) DOXPRI (XhoI) are shown and the size of the resulting fragments and the position of the probes are indicated (primers used to amplify probes from genomic DNA template are provided in Table ST2). The respective blots are shown in Fig 2B and 3B respectively. Chromosomal positions of the genes are given with reference to the *T. gondii* GT1 genome assembly version 6 (ToxoDB.org). Open boxes display exons, grey boxes introns and the hatched boxes in (**B**) show to new exons identified by 5' RACE-PCR (see Fig S3 for additional sequence detail).

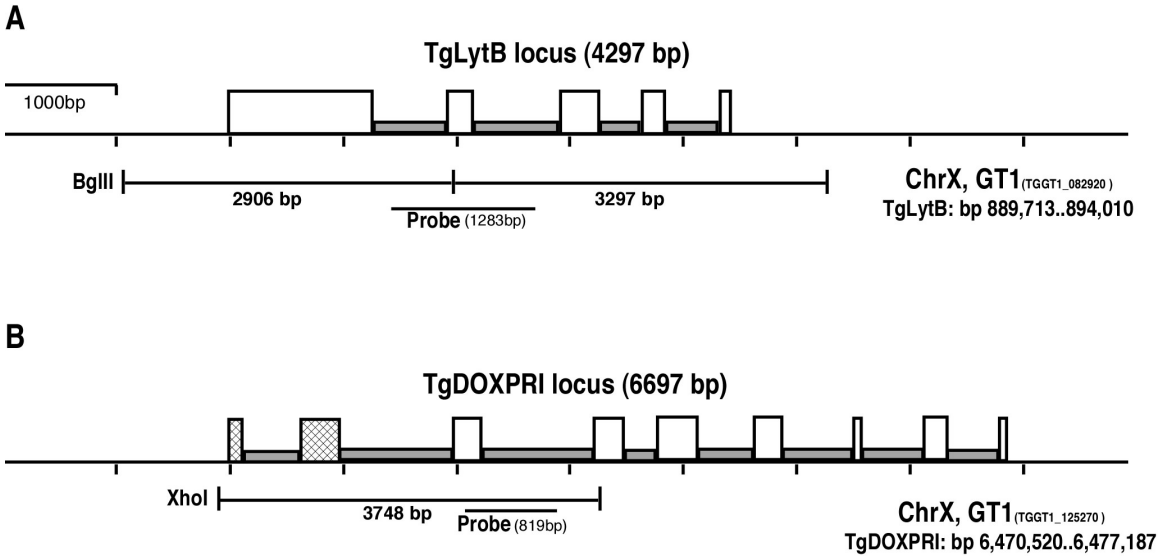


Figure 3.11: Multiple protein sequence alignment of *T. gondii* DOXPRI and apicomplexan and bacterial homologs. Clustal-W generated alignment of DOXPRI protein sequences from *Plasmodium falciparum* (Pf, PF14_0641), *Theileria annulata* (Ta, TA14290), *Toxoplasma gondii* (Tg, this study) and *Escherichia coli* (Ec, EU906103). The presumptive signal peptide of the *T. gondii* protein is underlined (the prediction is based on analysis using the SignalP algorithm). *T. gondii* DOXPRI protein sequence not previously predicted in the automated gene model but established by 5'RACE PCR is highlighted in bold type. Several functionally important sequence features of the well-studied *E. coli* enzyme (Mac Sweeney et al., 2005) are specifically indicated for comparison: the NADPH binding domain (blue), the central catalytic domain (green), the C-terminal α-helical domain (purple), active site flexible loop (brown), and the amino acids that make direct contact with the substrate are highlighted in red. ***** Identical residues; **:** conservative substitutions; **.** semi-conservative substitutions.

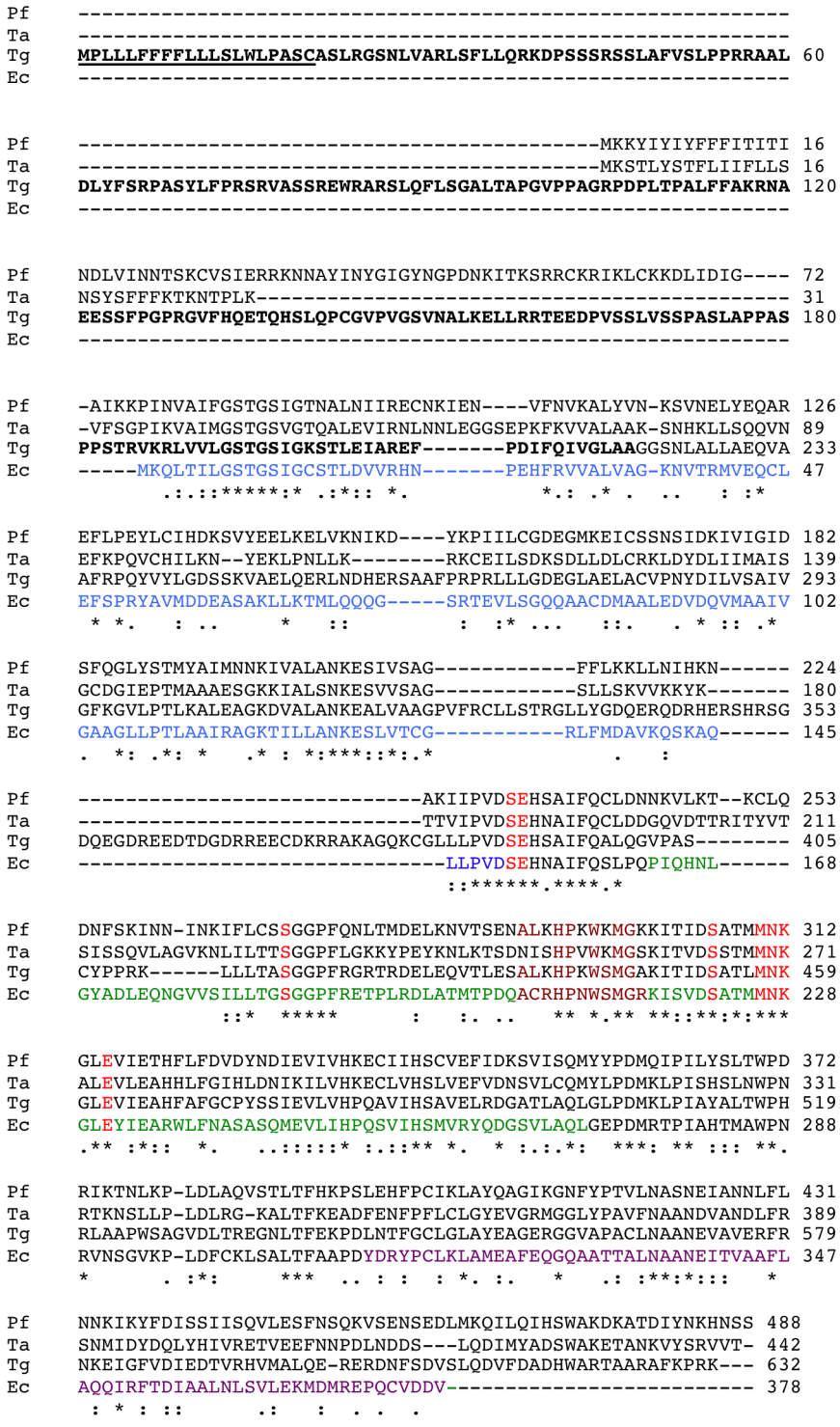

Pf ----------
Ta ----------
Tg MPLLLFFFFLLLSLWLPASCASLRGSNLVARLSFLLQRKDPSSSRSSLAFVSLPPRRAAL 60
Ec ----------
Pf ----------MKKYIYIYFFFITITI 16
Ta ----------MKSTLYSTFLIIFLLS 16
Tg DLYFSRPASYLFPRSRVASSREWRARSLQFLSGALTAPGVPPAGRPDPLTPALFFAKRNA 120
Ec ----------
Pf NDLVINNTSKCVSIERRKNNAYINYGIGYNGPDNKITKSRRCKRIKLCKKDLIDIG---- 72
Ta NSYSFFFKTKNTPLK---------- 31
Tg EESSFPGPRGVFHQETQHSLQPCGVPVGSVNALKELLRRTEEDPVSSLVSSPASLAPPAS 180
Ec ----------
Pf -AIKKPINVAIFGSTGSIGTNALNIIRECNKIEN----VFNVKALYVN-KSVNELYEQAR 126
Ta -VFSGPIKVAIMGSTGSVGTQALEVIRNLNNLEGGSEPKFKVVALAAK-SNHKLLSQQVN 89
Tg PPSTRVKRLVVLGSTGSIGKSTLEIAREF-------PDIFQIVGLAAGGSNLALLAEQVA 233
Ec -----MKQLTILGSTGSIGCSTLDVVRHN-------PEHFRVVALVAG-KNVTRMVEQCL 47
Pf EFLPEYLCIHDKSVYEELKELVKNIKD----YKPIILCGDEGMKEICSSNSIDKIVIGID 182
Ta EFKPQVCHILKN--YEKLPNLLK--------RKCEILSDKSDLLDLCRKLDYDLIIMAIS 139
Tg AFRPQYVYLGDSSKVAELQERLNDHERSAAFPRPRLLLGDEGLAELACVPNYDILVSAIV 293
Ec EFSPRYAVMDDEASAKLLKTMLQQQG-----SRTEVLSGQQAACDMAALEDVDQVMAAIV 102
Pf SFQGLYSTMYAIMNNKIVALANKESIVSAG------------FFLKKLLNIHKN------ 224
Ta GCDGIEPTMAAAESGKKIALSNKESVVSAG------------SLLSKVVKKYK------- 180
Tg GFKGVLPTLKALEAGKDVALANKEALVAAGPVFRCLLSTRGLLYGDQERQDRHERSHRSG 353
Ec GAAGLLPTLAAIRAGKTILLANKESLVTCG-----------RLFMDAVKQSKAQ------ 145
Pf ----------AKIIPVDSEHSAIFQCLDNNKVLKT--KCLQ 253
Ta ----------TTVIPVDSEHNAIFQCLDDGQVDTTRITYVT 211
Tg DQEGDREEDTDGDRREECDKRRAKAGQKCGLLLPVDSEHSAIFQALQGVPAS-------- 405
Ec ----------LLPVDSEHNAIFQSLPQPIQHNL------ 168
Pf DNFSKINN-INKIFLCSSGGPFQNLTMDELKNVTSENALKHPKWKMGKKITIDSATMMNK 312
Ta SISSQVLAGVKNLILTTSGGPFLGKKYPEYKNLKTSDNISHPVWKMGSKITVDSSTMMNK 271
Tg CYPPRK------LLLTASGGPFRGRTRDELEQVTLESALKHPKWSMGAKITIDSATLMNK 459
Ec GYADLEQNGVVSILLTGSGGPFRETPLRDLATMTPDQACRHPNWSMGRKISVDSATMMNK 228
Pf GLEVIETHFLFDVDYNDIEVIVHKECIIHSCVEFIDKSVISQMYYPDMQIPILYSLTWPD 372
Ta ALEVLEAHHLFGIHLDNIKILVHKECLVHSLVEFVDNSVLCQMYLPDMKLPISHSLNWPN 331
Tg GLEVIEAHFAFGCPYSSIEVLVHPQAVIHSAVELRDGATLAQLGLPDMKLPIAYALTWPH 519
Ec GLEYIEARWLFNASASQMEVLIHPQSVIHSMVRYQDGSVLAQLGEPDMRTPIAHTMAWPN 288
Pf RIKTNLKP-LDLAQVSTLTFHKPSLEHFPCIKLAYQAGIKGNFYPTVLNASNEIANNLFL 431
Ta RTKNSLLP-LDLRG-KALTFKEADFENFPFLCLGYEVGRMGGLYPAVFNAANDVANDLFR 389
Tg RLAAPWSAGVDLTREGNLTFEKPDLNTFGCLGLAYEAGERGGVAPACLNAANEVAVERFR 579
Ec RVNSGVKP-LDFCKLSALTFAAPDYDRYPCLKLAMEAFEQGQAATTALNAANEITVAAFL 347
Pf NNKIKYFDISSIISQVLESFNSQKVSENSEDLMKQILQIHSWAKDKATDIYNKHNSS 488
Ta SNMIDYDQLYHIVRETVEEFNNPDLNDDS---LQDIMYADSWAKETANKVYSRVVT- 442
Tg NKEIGFVDIEDTVRHVMALQE-RERDNFSDVSLQDVFDADHWARTAARAFKPRK--- 632
Ec AQQIRFTDIAALNLSVLEKMDMREPQCVDDV---------- 378

Figure 3.12: Expression of L-GlpT confers fosmidomycin sensitivity.

The growth of GlpT expressing RH-YFP parasites was tested by plaque assay in the presence and absence of 100 mM fosmidomycin. Note absence of plaques under drug. For comparison see Figure 4D, which shows robust plaque formation in the presence of 100 mM fosmidomycin for strains that do not express GlpT

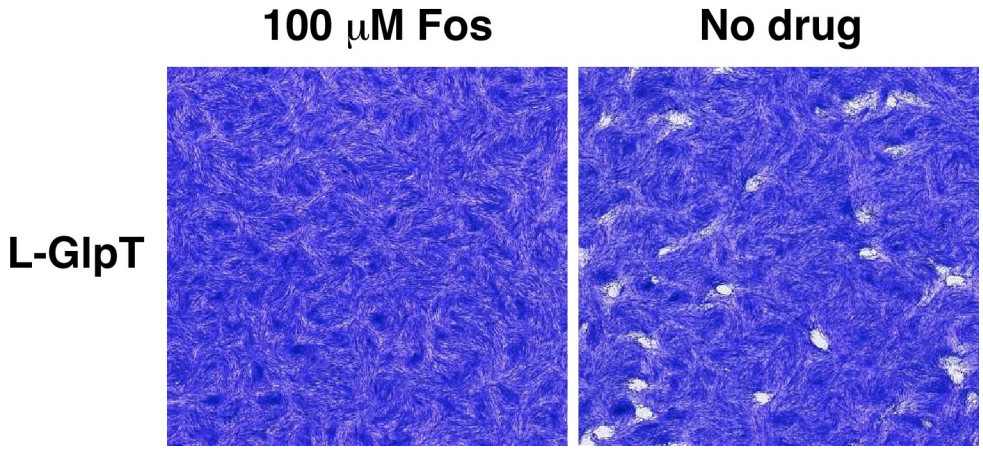


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Figure 3.13: Point mutation does not affect L-GlpT localization.

Immunofluorescence assays showing clones of RH-YFP parasites expressing L-GlpT$_{R45K}$ (top) or L-GlpT$_{R45KR269K}$ (bottom). The proteins are detected using the anti-Ty-1 antibody (Brookman et al., 1995) shown in the red channel, the green channels shows cytoplasmic YFP. Note membrane staining as described in Fig. 6 for wild type L-GlpT

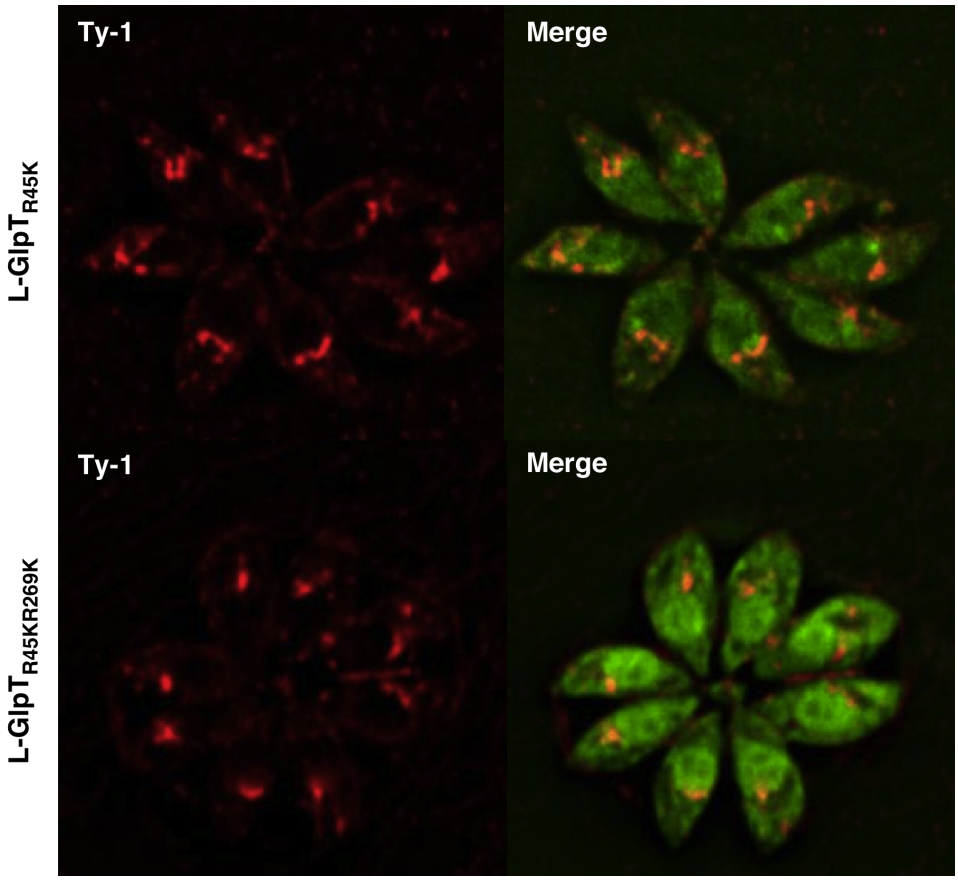

Figure 3.14: Loss of DOXPRI produces a more severe growth phenotype than loss of ACP. Conditional mutants in ACP (FASII (Mazumdar et al., 2006)), DOXPRI (isoprenoid), and APT (carbon import, (Brooks et al., 2010)) were tested by plaque assay in the presence (+ATc) or absence (-ATc) of anhydrotetracycline. Cultures were grown for 10 days prior to fixation and staining. Note that loss of DOXPRI mirrors the severity of loss of APT

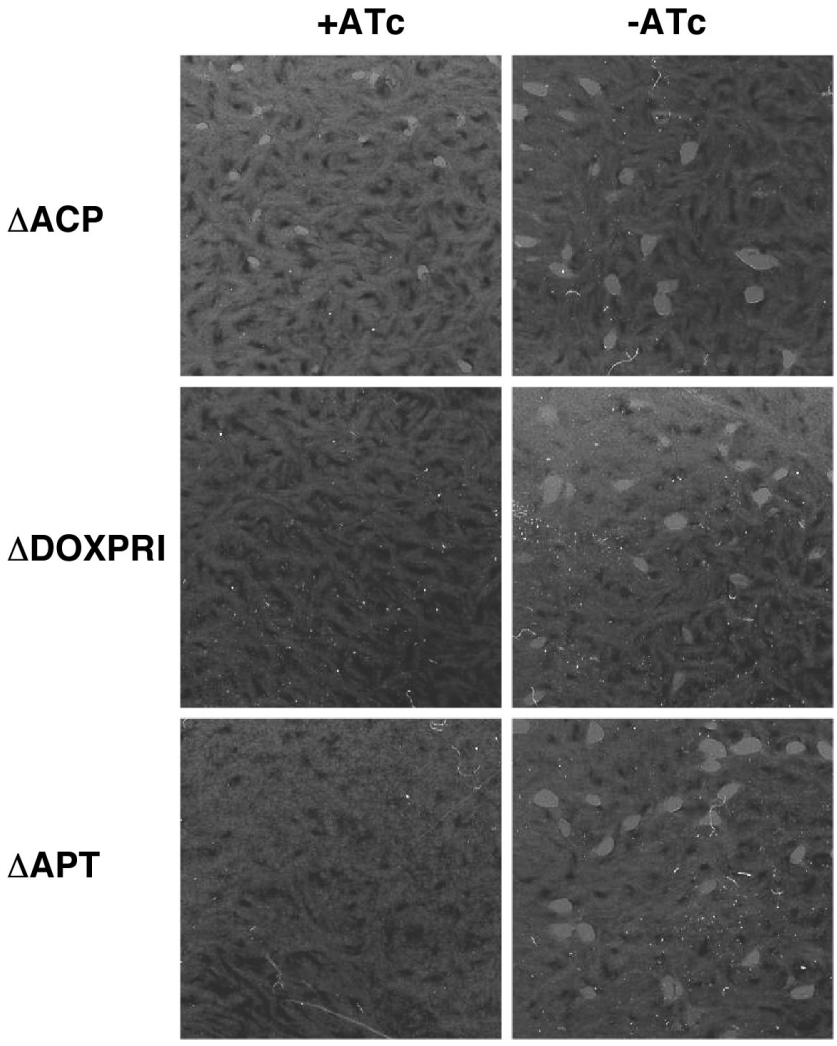

Table 3.1 : IC_{50} values for fosmidomycin and fosmidomycin-ester derivatives established for wild type and L-GlpT parasites.

***T. gondii* strain**	**Drug and IC_{50} value[1]**			
	fosmidomycin	diphenyl-fosm.	di-4methoxy-phenyl-fosm.	di-pivaloxyl-methyl-fosm.
RH/YFP-YFP	>100 μM[2]	>100 μM[2]	>100 μM[2]	32.7 μM
L-GlpT/YFP-YFP	1.6 μM	1 μM	2 μM	30.2 μM

[1]Growth was measured by fluorescence assay and IC_{50} values were established as detailed in Figure 7

[2]note that no precise IC50 value could be established due to resistance

Table 3.2: Primers used for construction of tagged genes for subsequent localization of their protein products

Name	Primers used for cloning with Restriction sites	Vector used for cloning	Method of localization	Localization
LytB	For_BamHI5'- TCGAGGATCCAAAATGCACACGAGAGGCGAAATGCAGATG Rev_AvrII5'- CTAGCCTAGGAGAGTCTCGGGCGACCAGCGTCT	pDT7S4M3	Cloned and introduced as minigene	Apicoplast
DOXPRI	For_BglII5'- ATCGAGATCTAAAATGAATCTTCTTCTCTTCTTCTTCTTTTTG Rev_AvrII5'- CGATCCTAGGCTTCCTCGGCTTAAAGGCCCTCGCT	pDT7S4M3	Cloned and introduced as minigene	Apicoplast
FNR-L	For_BglII 5'- GGAAGATCTATGGTTCGGGGCATCCGTCCTCGT Rev_AflII 5'- ATGCCTTAAGGGATGTTTGGTCGGTCGGGGTCG	pBTTy		
E.coli DOXPRI	For_NdeI 5'- ATGCCATATGAAGCAACTCACCATTCTGGGCTCG Rev_AvrII 5'- ATCGCCTAGGGCTTGCGAGACGCATCACCTCTTTTC	pBTTY-FNR_L	Cloned and introduced as minigene	Apicoplast
FNR-SP	For_KasI 5'- ATCGGGCGCCCGAACTTTGTACGAGCGCAGTCAG Rev_AflII 5'- ATCGCCTAAGGGATATAGCCACAGAGGCTACAAC	pBTTY		
E.coli GlpT	For_AflII 5'- ATGCCTTAAGATGTTGAGTATTTTTAAACCAGCGC Rev_AvrII 5'- GCATCCTAGGGACTCCGTTGCGTTCTTGCAG	pBTTY-FNR_L pBTTY-FNR_{SP}	Cloned and introduced as minigene	ER, parasite plasma membrane

Table 3.3: Primers used for gene deletions and Southern probes

Primers used to recombineer gene targeting cosmids (50 bp recombination sequences are shown in upper case)		**Primers used for Southern probes**
DOXPRI KO	For5'- GTCTGTCCCCTTCTTTTGCCGCAGCTCTTCTCTCCTCCATCCCAGCCACGcctcgactacggcttccattggcaac. Rev5'- TCATGAACTTACGCATCCCCTTTTGAATATGTGTCGGGGATTTTGCGTGTatacgactcactatagggcgaattgg	For5'- gcagcatcgggaagagcacattgg. Rev5'- cagcagattcggacaacataccagtg.
LytB KO	For5'- ATGACACGCCGCAATTAGATTCGCTTTCCAAGATGCACACGAGAGGCGAAggggcctcgactacggcttccattg Rev5'- CAGGCCGAACCTCATTATTTCAAAACTGAATAAACAGGGAGGGGGCCTATttgtaatacgactcactatagggcg	For5'- atacgggtggacgctgactc. Rev5'- caatgccttgcttcagtggag.

Table 3.4: Construction of DOXPRI expression vectors for immunization (DOXPRI mature) and enzymatic assay (MBP-DOXPRI+TP and MBP-DOXPRI mature)

Name	**Primers used for amplifying cosmid recombination fragment**	**Vector used**
DOXPRI mature	for 5'- TACTTCCAATCCAATTTAATGCAAGAGACTTGTGGTTTTGGGAAGCA rev 5'- TCCTCCACTTCCAATTTTAGCAAGCTTTCACTTCCTCGGCTTAAAGGCCCTC.	pAVA421, Cloned by LIC
MBP-DOXPRI+TP	For_EcoRI 5'- GCGAATTCTGCGCTTCTCTGAGGGGTTCGAACC Rev_HinDIII 5'- CCAAGCTTTCACTTCCTCGGCTTAAAGGCCCTC.	pMAL-C2X
MBP-DOXPRI mature	For_EcoRI5'- GCGAATTCAAGAGACTTGTGGTTTTGGGAAGCA Rev_HinDIII 5'- CCAAGCTTTCACTTCCTCGGCTTAAAGGCCCTC.	pMAL-C2X

CHAPTER 4

CONCLUSIONS

The phylum Apicomplexa contains important pathogens responsible for life threatening infection in humans and animals. These parasites strategize to survive in extreme conditions of parasitism and henceforth have survived efforts of intervention through ages. Hence there is a constant demand for the development of new drugs against these pathogens. Most members of the phylum have a plastid like organelle that these parasites acquired from a photosynthetic eukaryote. Although photosynthetic energy likely has been the initial impetus for the acquisition, many of the successive lineages including Apicomplexa retained the organelle despite the loss of photosynthesis. The most conceivable reason for the retention of this organelle in this phylum is the metabolic potential associated with it. The divergent nature of these metabolic pathways has also boosted the efforts to develop novel drugs targeting this organelle. Some of the drugs targeting these pathways have been quite effective in combating the infection and have even been used clinically to treat infection. One such drug is fosmidomycin, an antibiotic that targets the isoprenoid pathway in the apicoplast. Although the target pathway of fosmidomycin is the most conserved function of the apicoplast among the members of the phylum, many members of the phylum are naturally resistant to fosmidomycin. Using *Toxoplasma* as a model organism, I have

tried to dissect this important question. The important conclusions drawn from the study are.

THE APICOPLAST DOXP PATHWAY IS AN ESSENTIAL PATHWAY AND A VALID TARGET FOR CHEMOTHERAPY.

The first conclusion that we infer from our study is that the apicoplast DOXP pathway is essential in *T. gondii*. To reach that conclusion, we created two conditional mutants of the enzymes involved in the pathway using a conditional expression system. This strategy is a two step process 1) the expression of an exogenous minigene copy of the gene of interest under tetracycline regulatable promoter, selecting a parasitic clone for its expression and regulation of its expression using anhydrous tetracycline 2) targeting of the endogenous loci in this parasitic clone by homologous recombination and selecting for recombination using a drug selectable marker (Brooks et al., 2010). The clone with the native loci replaced is first identified through PCR and the replacement is then confirmed by southern blotting. The essentiality of the gene is studied by adding anhydrous tetracycline to shut off the regulatable copy. Using a cosmid based system (Brooks et al., 2010), we created two conditional mutants in the pathway, one in LytB, the enzyme catalyzing the last step of the pathway and then DOXPRI, the target of fosmidomycin that catalyses the initial rate limiting step. Mutations of the both these enzymes resulted in severe growth defect in parasites. These double mutations in the last and the initial rate limiting step also ruled out any metabolic shunting happening to the pathway from other sources.

RESISTANCE AGAINST FOSMIDOMYCIN IS DUE TO THE LACK OF DRUG UPTAKE

Since the target of the drug fosmidomycin was essential for the parasite, next we analyzed if the target enzyme is inherently resistant to fosmidomycin. To answer that, we complemented the *Toxoplasma* DOXPRI mutation with a fosmidomycin sensitive bacterial enzyme. This bacterial enzyme was targeted to the apicoplast using an apicoplast leader peptide. The bacterial enzyme retained the expression in the presence of anhydrous tetracycline, while the *Toxoplasma* enzyme expression was turned off. Hence, this parasitic strain is dependant on a sensitive bacterial enzyme in the presence of anhydrous tetracycline. Although, this bacterial enzyme complemented the function of the native enzyme as visualized by robust growth in plaque assay in the presence of anhydrous tetracycline, parasites remained resistant to fosmidomycin suggesting that lack of drug access as the reason for resistance.

HOST CELL MEMBRANE IS NOT THE CHIEF BARRIER FOR FOSMIDOMYCIN ENTRY

The chief barrier for the entry of fosmidomycin could either be the infected host cell membrane, parasitophorous vacuolar membrane, parasite plasma membrane or the apicolast membranes. To understand the most important barrier for fosmidomycin entry, we first looked at the role of host cell plasma membrane. Fosmidomycin sensitivity in

Plasmodium has been primarily assessed in the red blood cell stages of infection. The anucleated red blood cells are bags of haemoglobin, which are heavily modified by infected parasites. Sensitivity of this stage of infection to fosmidomycin could be attributed to easy drug access because of these modifications. To rule out that possibility, we tested the effect of fosmidomycin on *Plasmodium* liver stages of infection. The liver stages were equally sensitive as the red blood stages suggesting very limited role for red blood cell modifications by *Plasmodium* in drug access. To confirm the limited role of host cells directly in *Toxoplasma*, we tested esters on fosmidomycin on *Toxoplasma* infected cells using a standard growth assay. These esters have their polar groups esterified using non-polar lipophilic moieties to improve host cell permeability. Once inside the host cells, the ester groups will be cleaved off to release active fosmidomycin. But these esters were equally ineffective against *Toxoplasma* just like unmodified fosmidomycin confirming the limited role of host cell in drug access.

PARASITE PLASMA MEMBRANE IS THE CHIEF BARRIER OF FOSMIDOMYCIN ENTRY

The parasitophorous vacuolar membrane has been shown to be permeable to small molecules and hence the ther two formidable barriers for fosmidomycin entry could be the parasite plasma membrane or the apicoplast membranes. To understand the role of these barriers in fosmidomycin entry, we expressed a bacterial glycerol-3-po4 transporter capable of importing fosmidomycin in wild type parasites. Two versions of this transporter was expressed in parasites, one that carries a signal peptide at the N'

terminus to carry it to the parasite plasma membrane and other with an apicoplast leader peptide to carry it to the apicoplast membranes. The expression of both versions of the transporter made the parasites sensitive to fosmidomycin at concentrations similar to sensitivity observed in *Plasmodium* and *Babesia.* This sensitivity was completely reverted in parasites expressing transporter with mutations in two critical arginine residues known to be required for taking up the drug. Immunofluoroscent staining revealed robust staining of the parasite plasma membrane, when expressed with signal peptide and with apicoplast leader peptide. Surprisingly, the internal staining observed upon expression with a known apicoplast leader did not colocalize with the apicoplast. To confirm the fact that the transporter expressing parasites is accumulating fosmidomycin, we developed a classical agar zone of inhibition assay using *E.coli* DH5α bacterial cells grown in LB agar. In this assay, known amounts of fosmidomycin were initially added on to small paper discs and this was then layered on top of overnight culture of bacterial cells. The zone of inhibition around this discs were measured and a standard graph was made. Then, wild type parasites and transporter expressing parasites were then incubated with 100 μM fosmidomycin for 6 hours, pelleted down, washed, resuspended in 100ul water and then sonicated to lyse out the cells. This lysate was then added on to paper discs and layered on top of an overnight bacterial culture in LB agar. While the lysate from transporter expressing parasites inhibited the growth of bacteria as visualized by the zone of inhibition around the discs, wild type parasites could not suggesting that only transporter expressing parasites could accumulate fosmidomycin. We quantified this accumulation using the standard graph

made with known amounts of fosmidomycin. To confirm the fact that this accumulation is happening in the cytoplasm due to the plasma membrane expression of the transporter, we modified this assay by treating the parasites with 0.1% digitonin after incubation with fosmidomycin. The treatment will selectively permeabilize the contents of the cytoplasm while retaining the contents of the apicoplast lumen. The cytoplasmic contents released after digitonin treatment were loaded on to paper discs and layered on LB agar bacterial culture .The apicoplast pellet remaining was also sonicated as before and the lysate was also added to paper discs. While most of the fosmidomycin (~86%) was released by digitonin treatment as seen by the robust zone of inhibition, apicoplast lumen also retained some portion (14%). This confirmed the fact that the plasma membrane is indeed the chief barrier for fosmidomycin entry as most of the fosmidomycin accumulated in the parasite cytoplasm. This also suggested that resistant parasites lack the transporter needed for uptake. More importantly, the results implicated that sensitive parasites like *Plasmodium* will be having a presumptive transporter associated with the plasma membrane for uptake and mutations in the same can result in resistance.

ISOPRENOID BIOSYNTHESIS COULD BE THE CHIEF FUNCTION OF THE APICOPLAST.

Isoprenoid biosynthetic pathway is one of the most conserved functions of the apicoplast among the members of the phylum Apicomplexa. In *Toxoplasma*, a phenotypic comparison of the mutations associated with the isoprenoid pathway, fatty

acid pathway and apicoplast phosphate transporter showed that the phenotype associated with isoprenoid pathway mutation and the apicoplast transporter mutation behaved similarly in a standard plaque assay looking at growth. It also suggested that isoprenoid precursor biosynthesis could be a more essential function of apicoplast than the fatty acid biosynthesis in *Toxoplasma*. The conservation of the apicoplast DOXP pathway across the phylum and its essentiality in studied organisms suggest that this could be the chief function of the apicoplast.

FUTURE DIRECTIONS

The results of our study indicate the presence of a presumptive transporter involved in the import of fosmidomycin in sensitive parasites like *Plasmodium* and *Babesia*. The transporter should have similar function to the well-characterized glycerol-3-po4 transporter in bacteria known to import fosmidomycin. The hypothesis also assumes that, sensitive parasites like *Plasmodium* will be able to take up labeled glycerol-3-po4 while *Toxoplasma* as well as other resistant parasites will not be able to do so. We are currently investigating the ability of the resistant as well as sensitive parasites to uptake glycerol-3-po4. This will give one of the first clues in identifying the potential transporter involved in fosmidomycin uptake.

Our early analysis indicates that isoprenoid pathway is one of the most critical functions of the apicoplast. I have reviewed all the potential roles of the pathway in the chapter two of this dissertation. Our first priority is to investigate the role of apicoplast in maintaining mitochondrial homeostasis. Mutations associated with the apicoplast

phosphate transporter result in the loss of mitochondrial membrane potential and this loss was not associated with the loss of just fatty acid synthesis in the apicoplast. Hence this observation could be due to the loss of isoprenoid biosyntheis of the apicoplast. One potential reason could be that isoprenoid pathway is needed for the prenylation of ubiquinone, an important member of the mitochondrial electron transport chain. We are studying this hypothesis through genetic as well biochemical approaches. Another important function of the isoprenoid pathway is the need for the modification of tRNA's encoded by the apicoplast genome. One potential function of this modified tRNA could be to help read through premature stop codons in the reading frames of the message encoded by the apicoplast genome. These modified tRNAs could also be used for the biosynthesis of cytokinins. We already know that the enzyme involved in this modification called the tRNA isopentenyl transferase localizes to the apicoplast of Toxoplasma. The role of this modification process in the translation process as well as generation of cytokinins is also under study.

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