Study of the Apicoplast Biology in *Plasmodium falciparum* during Erythrocytic Schizogony

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I declare that I only used the sources and materials cited in the work, and that all citations, whether word for word or paraphrased are given as such.

I declare that I adhered to the guidelines set forth by the University of Tübingen to guarantee proper academic scholarship (Senate Resolution 25.05.2000).

I declare that these statements are true and that I am concealing nothing.

I understand that any false statements can be punished with a jail term of up to three years or a financial penalty.

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SUMMARY

Based on its prokaryotic nature, the apicoplast in *Plasmodium falciparum* is a unique target with great potential for generation of both chemotherapeutic and immunization intervention strategies against malaria. It is therefore essential to understand its biology. This study used a drug based approach to explore new aspects of apicoplast biology.

In part 1 of this study the established antibiotic clindamycin was used to study biogenesis and function of the apicoplast during erythrocytic schizogony. The data in this study showed that during erythrocytic schizogony, clindamycin inhibits the egress machinery of the parasite by inhibiting the biogenesis and function of the apicoplast. Interestingly, in order to egress from erythrocyte, these parasites rely on an unusual early supplementation with isopentenyl pyrophosphate or zaprinast. The data in this study further showed that, the apicoplast in *P. falciparum* is required during egress by playing a role in the secretion of proteins required for egress. In this way, this study expands the current understanding of the biology of the apicoplast and the mode of action of this antibiotic during erythrocytic schizogony.

In part 2 of this study, experimental antibiotics called acyldepsipeptides (ADEPS) were tested to determine whether they can target the biogenesis or function of the apicoplast in *P. falciparum* during erythrocytic schizogony and therefore whether they can be used as antimalarial drugs. The data in this study shows that ADEPS are able to inhibit growth of *P. falciparum*. On one hand, in parasites containing the apicoplast they appear to inhibit its biogenesis and or function. On the other hand, ADEPS also inhibit the growth of *P. falciparum* parasite lacking the apicoplast. Consequently, in *P. falciparum*, ADEPS appear to have apicoplast dependent targets and apicoplast independent targets. Never-the-less, ADEPS appear to be active against *P. falciparum* and therefore they can be invoked as antimalarial drugs. This study therefore contributes to the expansion of the arsenal to combat malaria.
ZUSAMMENFASSUNG


Im zweiten Teil der Studie wurde untersucht, ob experimentelle Antibiotika, genannt Acyldepsipeptide (ADEPS), die Biogenese oder Funktion des Apicoplasten während der erythrozytären Schizogonie hemmen können und sich daher als Medikament gegen Malaria eignen würde. Es stellte sich heraus, dass ADEPS zwar das Wachstum von P. falciparum hemmen können, egal ob die Parasiten ein Apicoplast haben oder nicht. Dass heißt, in P. falciparum, gibt es zwei gruppe als Angriffsziele von ADEPS: das eine wo der Apicoplast abhängig ist und das andere wo der Apicoplast unabhängig ist. Aufgrund der bestehenden Wachstumshemmung können sie allerdings trotzdem als Medikamente gegen Malaria eingesetzt werden.
LIST OF ABBREVIATIONS

ACP.............Acyl Carrier Protein
ACTs...............Artemisinin - based Combination Therapies
ADEPS............Acyldepsipeptides
BAS................Blocking Aid Solution
BSA..............Bovine Serum Albumin
Ca^{2+}..........Calcium ions
cDNA............Complementary DNA
CDPK5..........Calcium Dependent Protein Kinase 5
ClpP.............Caseinolytic Protease
CRT..............Chloroquine Resistance Transporter
DHFR............Dihydrofolate Reductase
DHPS............Dihydropyrimidate Synthase
DNA...............Deoxyribonucleic acid
DMSO............Dimethyl Sulfoxide
E64..............trans - Epoxysuccinyl - L - leucylamido (4 - guanidino) butane
Fe................Iron
FACS...............Fluorescence activated cell Sorter
FITC...............Fluorescein Isothiocyanate
FSC...............Forward Scatter
g..................gram
I145TP..........Inositol 1,4,5 triphosphate
IPP..............Isopentenyl Pyrophosphate
IFA...............Immunofluorescence Assay
Kb................1,000 Nucleotide Bases
l..................Liter
n..................Nano
m..................Metre
M..................Molar
ml................Milliliter
Mb................Mega Base
MFI................Mean Fluorescence Intensity
MSP...............Merozoite Surface Protein
µ......................micro
PBS..................Phosphate Buffer Saline
PCR..................Polymerase Chain Reaction
Pfatl..................P. falciparum Arginine-tRNA ligase
PfMDR1..............P. falciparum Multi Drug Resistance 1
PfPLPs..............P. falciparum perforin like proteins
PfSUB1.............Plasmodium falciparum subtilisin like serine protease-1
Pl(3)P..............Phosphatidylinositol 3 Phosphate
Pl(4)P..............Phosphatidylinositol 4 Phosphate
Pl 4,5 BP..........Phosphatidylinositol 4,5 Bisphosphate
PKG...............Protein kinase G
RNA...............Ribonucleic Acid
RT-PCR............Real Time - Polymerase Chain Reaction
S.....................Sulphur
SERAs..............Serine Like Antigens
SSC...............Side Scatter
tRNA..............Transfer Ribonucleic Acid
WHO..............World Health Organization
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INTRODUCTION

Malaria

The phylum *Apicomplexa* comprises of unicellular obligate endoparasites. These organisms have a characteristic cellular organelle called the apical complex. This organelle is employed for the invasion of the host cell. Members of this group include *Toxoplasma, Isospora, Cryptosporidium, Cyclospora, Babesia, Eimeria* and *Plasmodium*. These parasites cause toxoplasmosis, isosporiasis, cryptosporidiosis, cyclosporiasis, babesiosis, coccidiosis and malaria respectively.

Malaria continues to be a global health challenge (Figure 1). Basing on the world health organisation (WHO) report, during the year 2015, 214 million people were infected with malaria and 438,000 died from malaria (WHO 2015 A).

![Figure 1 Global malaria transmission (Source: WHO 2014 A). Malaria is predominantly concentrated along the tropical countries. Most of this burden lay in Africa (WHO 2015).](image)

*Plasmodium falciparum* life Cycle

Malaria is a disease that results from infection with *Plasmodium*. Five species of *Plasmodium* can infect human beings, namely: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* (Stephens 1914; Chin *et al.*, 1968; Escalante *et al.*, 1995; Escalante and Ayala 1995). Among these species, *P. falciparum* is the most dangerous (WHO 2015).

*P. falciparum* is an intracellular parasite and its life cycle (Figure 2) occurs in the
female *Anopheles* mosquito and human being. The infection starts from a bite by an infected mosquito. The respective parasite stage known as sporozoite enters the bloodstream. It then proceeds to the liver and invades hepatocyte. Here it initiates a phase of its life cycle called liver stage which is characterized by parasite transformation and asexual multiplication. This produces thousands of daughter cells known as merozoites contained in a structure called merosome. Merozoites get released from the infected liver cell into the blood stream in a process called egress. Here they bind to and invade erythrocytes initiating another phase of the parasite’s life cycle called erythrocytic schizogony i.e. the blood stage.

Within the erythrocytes, the parasite undergoes transformation also into different forms called rings, trophozoites and eventually into schizonts. During the schizont stage, the parasite undergoes asexual multiplication resulting in about 10-32 merozoites. These merozoites are in-turn released from the infected erythrocytes also through the egress process. They then invade uninfected erythrocytes to further growth of the parasite. It is the continuous rupturing of the erythrocytes which interferes with their physiological function leading to the clinical symptoms associated with malaria disease (Lodish *et al.*, 2003). Some merozoites transform into gametocytes which are then taken up by the mosquito during a blood meal. In the mosquito, gametocytes egress from erythrocytes, reproduce sexually eventually generating thousands of sporozoites contained within an oocyst in the midgut of the mosquito. These sporozoites in turn also egress in order to continue the parasite’s life cycle.
Figure 2 Malaria life cycle (Source: Cowman et al., 2012). Once an infected mosquito bites a person sporozoites enter the bloodstream and migrate to the liver. Here the parasite undergoes transformation and multiplication giving rise to merozoites that infect erythrocytes. Here the parasite undergoes again transformation and multiplication producing more merozoites. The parasite transforms also into sexual forms known as gametocytes which are taken up by a mosquito during a blood meal. There they undergo the sexual phase of the parasite life cycle. This in turn produces sporozoites which continue the parasite’s life cycle.

**Adaptation of *P. falciparum* during the blood stage**

During the blood stage the parasite has evolved some interesting mechanisms that promote its survival within erythrocytes, which have been exploited for experimental strategies to understand the biology of the parasite and generation of intervention strategies. Some of these mechanisms lay the basis of my key experimental procedures.

The merozoite has a size of about 1.2 μm (Boyle et al., 2010 B). It has a surface coat, cytoskeleton and organelles. The organelles are: nucleus, mitochondrion, ribosomes, endoplasmic reticulum, Golgi apparatus and the apical complex. The apical complex contains a number of secretory organelles which include rhoptries,
micronemes, exonemes and dense granules (Cowman et al., 2012).

During the invasion process, the parasite has to first make contact with the erythrocyte before penetrating it. This requires a parasite ligand called merozoite surface protein (MSP) to bind to receptors on the erythrocytes. This interaction can be inhibited by heparin thereby preventing invasion (Boyle et al., 2010 A).

After penetrating the erythrocytes, it then uses the host cell membrane to form a parasitophorous vacuole, which houses the parasite (Lingelbach and Joiner 1998). As the parasite starts to mature and grow, it exports proteins to the host cell and remolds the host cell plasma membrane. These play important roles in nutrient acquisition and protection against the immune system (Lodish et al., 2003; Goldberg and Cowman 2010). Because these membrane remodeling are extensive in older parasite stages and absent in rings, exposure of the parasites to 5% D-Sorbitol can be used to get rid of trophozoites and schizonts (Lambros and Vanderberg 1979).

The parasite salvages proteins from the erythrocytes by actively importing haemoglobin and enzymatically digesting it in the food vacuole. Heme as a toxic by-product of this process is converted to non toxic hemozoin (Olliaro and Goldberg 1995). Iron as a building block of heme can be exploited to magnetise the trophozoites and schizonts, thereby separating them from rings and uninfected erythrocytes which lack the food vacuole (Paul et al., 1981).

The parasite has a genome of 23 Mb distributed over 14 chromosomes (Gardner et al., 2002). During the blood stage it reproduces asexually by schizogony. This is a unique type of mitosis in which the parasite undergoes several rounds of unsynchronised mitotic division within the mother cell. This is later followed by a uniform final round of mitotic division and assembly of the daughter cells (Francia and Striepen 2014).

This asynchronous division yields about 10 – 32 merozoites. In order to egress the host cell, the parasite degrades the parasitophorous vacuole membrane and the erythrocytes membrane. Since this process requires cysteine proteases, it can therefore be inhibited by the compound trans - Epoxysuccinyl - L - leucylamido (4 - guanidino) butane (E64, from Sigma aldrich). This inhibition trap the merozoites within the host cell (Salmon et al., 2001). These merozoites can then be released and purified by mechanically rupturing the schizonts by filtration through a 1.2 µm
membrane syringe filter (Boyle et al., 2010 B).

**Malaria intervention strategies**

Malaria can be prevented or cured. Since the year 2000, owing to intervention strategies there has been a reduction of 18% and 48% in the morbidity and mortality resulting from malaria respectively (WHO 2015). Prevention of transmission is done through vector control using long lasting insecticide treated bed nets and in door residual spraying (WHO 2015). Treatment is by the use of antimalarial drugs and it would also ideally involve vaccination, but upto now no malaria vaccine has been licensed (WHO 2015).

**Antimalarial drugs**

There are various antimalarial drugs with different modes of action. These can be broadly classified into the following groups: aminoquinolines, arylaminoalcohols, artemisinines, antifolates, inhibitors of the respiratory chain and antibiotics (Schlitzer 2008). Aminoquiolines include 4-aminoquinolines e.g. chloroquine and 8-aminoquinolones e.g. primaquine. During the blood stage, the parasite digests hemoglobin in its food vacuole. This process produces heme, which is toxic to the parasite. Heme is subsequently converted to hemozoin, in order to neutralize it (Olliaro and Goldberg 1995). 4-aminoquinolines act by inhibiting this process. Arylaminoalcohols inhibit digestion of hemoglobin. Drugs in this group are quinine, mefloquine, halofantrine and lumefantrine. Artemisinins include artemether and artesunate. These drugs act by first being transformed into dihydroartemisinin, which is the active molecule (Luo and Shen 1987). This has endoperoxide bonds, which upon reaction with iron produce radicals that are toxic to the parasites (Meshnick et al., 1993). Antifolates act as inhibitors of dihydrofolate reductase (DHFR) or dihydropterate synthase (DHPS) in the folate biosynthesis pathway. They include: sulfadoxine, pyrimethamine, dapsone and chlorpoguanil. Inhibitors of the respiratory chain include atovaqoune (Schlitzer 2008). It acts by binding to cytochrome bc1 complex, thereby inhibiting the generation of mitochondrial membrane potential (Fry and Pudney 1992; Srivastava et al., 1997; Kessl et al., 2003; Schlitzer 2008).
Antimalarial drug resistance

Despite the availability of all of these antimalarial drugs, the malaria parasite has however developed various mechanisms which reduce their activities and therefore their application. Resistance to 4-aminoquinolines is known to result from mutations in the gene that encodes a protein called chloroquine resistance transporter (CRT). This reduces the activity of these drugs by removing them from the food vacuole (Fidock et al., 2000). Amplification of the gene encoding a transport protein known as *P. falciparum* multidrug resistance 1 (PfMDR1), has been associated with reduced activity of Arylaminoalcohols (Peel et al., 1994; Woodrow and Krishna 2006). Resistance to artemisinins is associated with parasite dormancy (Teuscher et al., 2010), a phenomenon attributed to an overactivated phosinositidyinositol - 3 - kinase (PI3K) and consequently phosphoinositydylinositol - 3 - phosphate metabolism (PI3P) (Mbegue et al., 2015). Resistance to antifolates is due to single point mutations in the genes that code for DHFR or DHPS (Triglia et al., 1997; Peterson et al., 1988). Resistance to atovaqoune occurs due to single point mutations in the gene that codes for cytochrome b (Walker et al., 1998).

How and why the parasite develops resistance is due to a number of different factors, namely: mutations in parasite, cross drug resistance, multiple drug resistance, pharmacokinetics, drug half-life, host immunity, transmission, vector and environment (Sinha et al., 2014). Initially, drug resistance starts due to spontaneous single or multiple point mutations that naturally occur. These mutations convert the genetic make up of wild type parasites. This in turn transforms them to mutants which do not respond to drug treatment in different ways: the mutations may change the affinity of the drug to its target. Or they may inhibit accumulation of the drug inside the parasite. These mutants in turn multiply giving rise to more drug resistant parasites. Emerged drug resistance may then become complicated by several factors. Parasites may become resistant to drugs belonging to the same family or having a similar mode of action. This is called cross drug resistance. Parasite may also become resistant to more than one antimalarial drug. This is known as multiple drug resistance. Owing to poor understanding of pharmacokinetics dynamics involving drug concentration vs parasite sensitivity or resistance, insufficient dosage may be used in the treatment of resistant parasites, thereby selecting them instead of clearing them, therefore, promoting their expansion. Some drugs have longer half lives. They therefore remain
in the circulation for a long time. This act as a form of drug pressure, which although may eliminate sensitive parasites, the concentration may not be sufficient to eliminate resistant parasites. Therefore, promoting their proliferation. The extent to which resistant parasites proliferate is also governed by the host immunity. This is because if the immune system is able to clear parasites resistant to antimalarial drugs, then they will not proliferate. On the contrary, if it is not able to clear them then they will proliferate. Immunity governs also the rate at which malaria symptoms develop. People from high malaria transmission areas e.g. Africa, develop partial immunity to malaria with time. They therefore, do not easily develop malaria symptoms as compared to people from low transmission areas like the southeast of Asia. Due to this reason antimalarial drugs are highly likely to be used alot in the latter. This can be a source of drug pressure in this region increasing the chances of selecting resistant parasites which can spread to the former region (Sinha et al., 2014).

Development of parasite resistance to antimalarial drugs limits the fight against malaria. The WHO therefore recommends the use of artemisinin-based combination therapies (ACTs) to combat malaria (WHO 2015). As the name suggests, in this strategy artemisinin drugs are combined with other antimalarial drugs. However, there are reports of resistance to artemisinins (Dondorp et al., 2009; Ashley et al., 2014; WHO 2014 B). As a back up measure, antibiotics in combination with other antimalarial drugs are recommended by the WHO as antimalarial drugs (WHO 2015 B) and they are currently being used against artemisinin or ACTs resistant parasites (Dondorp et al., 2009).

**Apicoplast**

**The origin of the apicoplast**

Except the species *Cryptosporidium* (Xu et al., 2004), parasites belonging to the phylum *Apicomplexa* have a plastid called the apicoplast (Lang-Unnasch et al., 1998). It was discovered a few decades ago (Kilejian A 1975; Borst et al., 1984; Wilson et al., 1996), to have originated through the process of secondary endosymbiosis (Lim and McFadden 2010). This process involved; a unikont which engulfed a cyanobacterium giving rise to a red algae. The latter was then engulfed by a bikont, followed by genetic rearrangements with the transfer of most of its DNA into the bikont (Lim and McFadden 2010) (Figure 3).
As a consequence of these endosymbiotic processes; the apicoplast genome was reduced to 35 Kb containing 64 genes (Wilson et al., 1996); it is surrounded by four membranes (Köhler S et al., 1997; McFadden and Roos 1999; McFadden 2010) and it relies on about 540 proteins that it has to import from the host cell (Waller et al., 1998). This imported proteins have to cross the four membranes surrounding it and this occurs by post-translationally targeting of these proteins to the apicoplast via a bipartite leader signal, that consists of a signal sequence and a transit signal (DeRocher et al., 2001; Waller et al., 2000). These signals function to aid the cotranslation importation of proteins into the endo-membrane system and sorting into the apicoplast respectively (Waller and McFadden 2005). Within the apicoplast, these proteins function in the expression of its genome (Waller et al., 1998) or in the metabolic pathways that it hosts (Ralph et al., 2004).

![Figure 3](image)

**Figure 3** Origin of the apicoplast (Source: Howe and Purton 2007). The apicoplast resulted from two endosymbiotic events between two eukaryotic cells and one prokaryotic cell followed by genetic rearrangements.
Functions of the apicoplast

The apicoplast hosts a number of metabolic pathways; the type II fatty acid synthesis, non-mevalonate isoprenoid synthesis, heme biosynthesis, Fe-S cluster biosynthesis, lipoate synthesis, tRNA modifications and ferredoxin redox system (Ralph et al., 2004). In return, products of these pathways are essential for the parasite. The apicoplast functions during the mosquito stage to produce heme (Nagara et al., 2013; Ke et al., 2014), during liver stage to produce fatty acids (Yu et al., 2008; Vaughan et al., 2009) and during the blood stage to produce isoprenoid precursors known as isopentenyl pyrophosphate (IPP) (Jomaa et al., 1999; Ye and DeRisi 2011; Wiley et al., 2015).

The products of the isoprenoid biosynthetic pathway (among other roles) are essential for post-translational modifications of proteins especially the anchorage to membranes (Alberts et al., 2008; Howe et al., 2013). Therefore, the apicoplast as an essential organelle is a validated drug target (Jomaa et al., 1999; Waller and McFadden 2005; Yu et al., 2008; Goodman et al., 2007; Ye and DeRisi 2011).

Biogenesis of the apicoplast

There is only one copy of the apicoplast in the Plasmodium parasite (Waller and McFadden 2005) and it is a very essential organelle (Jomaa et al., 1999; Ye and DeRisi 2011; Yu et al., 2008; Vaughan et al., 2009; Niagara et al., 2013; Ke et al., 2014; Wiley et al., 2015). Because it cannot be synthesised denovo, it has to be inherited from the mother cell. For this to happen, it undergoes biogenesis in the course of the parasite’s life cycle in hepatocytes, erythrocytes and in the midgut wall of the mosquito. It is then later segregated to the daughter cells towards the end of the cycle (Waller and McFadden 2005; Stanway et al., 2009).

How this occurs, is that, its biogenesis is coupled to the parasite transformation and cell division. During the early ring stage the apicoplast appears as a rod shaped and slightly curved structure. It then develops into a spherical structure. As the ring stage transforms into a trophozoite stage, it slowly increases in size but still remains as a spherical structure. It then grows, increases in length and branches forming a multi-branched structure in the transition from late trophozoite into early shizont stage. During this transition, the nucleus of the parasite undergoes mitosis to form multiple nuclei. Division of the apicoplast is delayed until this process is completed.
Therefore, it remains as a multibranched structure until late schizont stage. It is then segregated followed by cytokinesis of the parasite to form merozoites. These then egress, each carrying only one copy of the apicoplast (Waller and McFadden 2005).

The apicoplast as a target for antibiotics

The apicoplast has a 35 Kb genome (Wilson et al., 1996) and a number of metabolic pathways (Ralph et al., 2004). Due to its prokaryotic nature (Wilson et al., 1996), it is amenable to inhibition by antibiotics (Surolia et al., 2004; Burkhardt et al., 2007; Dahl and Rosenthal 2007; Goodman et al., 2007; Friesen et al., 2010). These antibiotics target the apicoplast differently (Waller and McFadden 2005; Goodman et al., 2007) and they can be classified into two groups: Those that inhibit house keeping functions and those that inhibit metabolic pathways.

Ciprofloxacin blocks DNA replication by inhibiting DNA gyrase (Divo et al., 1988). Rifampicin blocks transcription by inhibiting prokaryotic RNA polymerase (Calvori et al., 1965). Several other antibiotics inhibit protein translation e.g. doxycycline, tetracyclines, chloramphenicol, azithromycin, thiostrepton and clindamycin (Waller and McFadden 2005; Goodman et al., 2007). Doxycycline and tetracyclines act by binding to the prokaryotic 16S ribosomal RNA (Budimulja et al., 1997), while chloramphenicol, thiostrepton, azithromycin, and clindamycin act by binding to the prokaryotic 23S ribosomal RNA (Drainas et al., 1987; Pfefferkorn and Borotz 1994; Beckers et al., 1995; Tenson et al., 2003; Dharia et al., 2010).

Inhibitors of non-house keeping functions include: fosmidomycin which by targeting 1-Deoxy-D-xylulose-5-phosphate reductoisomerase inhibits the non mevalonate isoprenoid biosynthesis pathway (Jomaa et al., 1999); Triclosan and thiolactomycin which inhibit the type II fatty acid biosynthesis pathway by targeting FabI and FabF/H enzymes of this pathway respectively (Surolia and Surolia 2001; Waller et al., 2003).

By acting on different targets these antibiotics inhibit the parasite growth differently during the blood stage: ciprofloxacin, rifampicin and thiostrepton inhibit parasite growth within one cycle (Goodman et al., 2007). Tetracyclines and clindamycin inhibit parasite growth after two cycles, a phenomena termed delayed death (Surolia et al., 2004; Burkhardt et al., 2007; Dahl and Rosenthal 2007; Goodman et al., 2007).

The antibiotics clindamycin and azithromycin have been used to demonstrate that it
is possible to generate a whole organism based vaccine against malaria. Whereby, infecting mice with sporozoites and at the same time treating them with these antibiotics, the apicoplast biogenesis becomes inhibited. However, these parasites mature forming metabolically active merosomes, which provide a source of immunization against rodent malaria (Friesen et al. 2010).

**Clindamycin**

Clindamycin is an antibacterial drug belonging to lincosamide group of antibiotics. It is synthesised from the antibiotic lincomycin (Birkenmeyer and Kagan 1970; Spizek and Rezanka 2004). It acts by binding to the 23S ribosomal RNA in the large subunit ribosome in prokaryotes (Dharia et al., 2010). This prevents ribosomal translocation during protein synthesis. Consequently this leads to the inhibition of protein translation (Pfefferkorn and Borotz 1994; Camps et al., 2002; Tenson et al., 2003).

This effect of inhibiting protein synthesis has been shown to lead to inhibition of the apicoplast biogenesis (Dahl and Rosenthal 2007; Goodman et al., 2007). Consequently, the daughter cells formed within the mother cell do not get a copy of the apicoplast. This results in the inhibition of the parasite (Dahl and Rosenthal 2007; Goodman et al., 2007). However, this clindamycin-induced inhibition of the apicoplast biogenesis and parasite growth has been shown to occur differently during the parasite cycle:

- During the liver stage, exposure of clindamycin to sporozoites before invasion, results in the parasites developing into full maturity with no difference to untreated parasites. However, the biogenesis of the apicoplast in these parasites is inhibited (Friesen et al., 2010).
- In contrast, during the blood stage, exposure of clindamycin to the parasite post invasion results in normal parasite growth and apicoplast development. These parasites are then able to egress, invade uninfected erythrocytes but inhibition of the apicoplast biogenesis occurs. Consequently this leads to arrest of parasite growth (Surolia et al., 2004; Burkhardt et al., 2007; Dahl and Rosenthal 2007; Goodman et al., 2007).

Therefore, does the difference in the exposure of clindamycin before invasion during the liver stage and exposure post invasion during the blood stage, have an influence
on its effects on the observed differences; in the timing of the inhibition of the apicoplast biogenesis and parasite growth? In order to clarify this discrepancy, an ideal approach would be to set up a clear cut experiment during the blood stage by starting with the exposure of clindamycin to merozoites before invasion.

**Acyldepsipeptides**

Acyldepsipeptides (ADEPS) are a new type of antibacterial drugs produced from a parent molecule called A54556 complex (Michel and Kastner 1985; Brötz - Oesterhelt *et al.* 2005). In bacteria, ADEPS act via a unique mode of action (Brötz - Oesterhelt *et al.*, 2005). They bind to ClpP protease, activating it uncontrollably (Brötz - Oesterhelt *et al.*, 2005; Gersch *et al.*, 2014). The unregulated ClpP then preferentially targets bacterial cell division machinery (Brötz - Oesterhelt *et al.*, 2005; Sass *et al.*, 2011). This results in the inhibition of bacterial cell division. The parasite ends up as filamented or swollen cells (Sass *et al.*, 2011; Brötz - Oesterhelt *et al.*, 2005). Since the apicoplast is bacterial in nature (Wilson *et al.*, 1996) and has a ClpP protease too (Bakkouri *et al.*, 2010), therefore, can ADEPS target the apicoplast and be used as antimalarial drugs?

**Aim of the study**

The main aim of this study was to expand the understanding of the biology of the apicoplast as a unique target for development of antimalarial intervention strategies.

1. The first part of this study used the classical antibiotic called clindamycin to study the effects of synchronised antibiotic drug pressure and parasite cycle, to the biogenesis of the apicoplast and growth of *P. falciparum* during blood stage, and to follow-up the emerging phenotype by doing detailed studies.
2. The second part of this study used new types of antibiotics called Acyldepsipeptides (ADEPS), to determine whether they can inhibit the biogenesis and function of the apicoplast in *P. falciparum*, and therefore whether they can be used as antimalarial drugs.
MATERIALS AND METHODS

Materials

A. Equipment

1. Incubator (37°C)
2. Technical gas (92% N₂, 5% CO₂, 3% O₂)
3. Laminar flow hood
4. Centrifuge (Heraus Megafuge 1.0 R)
5. Light Microscope (Nikon Eclipse E200)
6. Electronic pipette dispenser (Integra Pippetboy)
7. Balance (Ohaus Adventurer Pro)
8. Magnetic stirrer (Heidolph MR3001)
9. Vacuum pump
10. Water bath (E5 Medingen)
11. Liquid nitrogen tank (ARPEGER 170)
12. Freezer (-80°C) (Heraus Hera freezer)
13. Freezer (-20°C) (Premium Liebherr)
14. Refrigerator (4°C) (Premium Liebherr)
15. pH metre (Schot Instruments Lab 850)
16. VarioMACS™ separation magnet system and columns (Miltenyi Biotec)
17. Facs machine (BD)
18. PCR machine (Eppendorf master cycler epgradient S)
19. qPCR machine (CR Corbert Research)
20. Nano drop (Nanodrop®)
21. Microwave (LG Intellowave)

22. Gel doc (Haiser R1, RS Dark reader Illuminator)

23. Fluorescence microscope (Zeiss Observer.Z1)

B. Material
   1. Cell culture flasks with filter bottle top (Greiner bio-one T25/T75)
   2. Sterile tubes (Greiner bio-one, 15 ml/50 ml)
   3. Sterile serological pipettes (Greiner bio-one, 1 ml, 2 ml, 5 ml, 10 ml, 25 ml)
   4. Glass Pasteur capillary pipettes (WU Mainz, long size, 230mm)
   5. Glass slides (Thermo Scientific, ground edges, frosted ends)
   6. Sterile filter units (Millipore, Steritop, 0.22 µm for 500 ml bottles)
   7. Sterile syringe filter (Roth, 0.22 µM)
   8. Sterile syringe (BD, 5, 10 and 20 ml)
   9. Cryo - vials (Greiner bio-one)
   10. 96 well plates (Greiner bio-one)
   11. Multichannel pipette (Eppendorf)
   12. Pipettes (Eppendorf Research)
   13. Aluminum paper foil (Universal)
   14. PAP Pen (Sigma Aldrich)
   15. Poly - L - Slides (Sigma Aldrich)

C. Reagents
   1. Purified washed erythrocytes type O+ (Blood bank)
   2. Human blood AB+ serum (Blood bank)
   3. RPMI (Invitrogen)
   4. Albumax II (Invitrogen)
5. Hypoxanthine (ccpro)
6. Gentamicin (Invitrogen)
7. Giemsa solution (Merck)
8. NaCl (VWR Prolabo)
9. Sorbitol (Fluka Biochemika)
10. Glycerol (Roth)
11. Giemsa solution (Merck)
12. Cell culture water
13. Tris base ()
14. HCl (Sigma Aldrich)
15. 0.5M EDTA (Acros organics)
16. DMSO (Grüssing Diagnostik analytika)
17. Saponin (Sigma Aldrich)
18. Triton X-100 (Sigma Aldrich)
19. SYBR Green I (Invitrogen)
20. 1.2 µm syringe filters (Sartorius)
21. PBS (Sigma Aldrich)
22. E64 (\textit{trans}-Epoxysuccinyl-L-leucylamido (4-guanidino) butane (Sigma Aldrich))
23. Paraformaldehyde (Applichem)
24. Gluteraldehyde (Sigma Aldrich)
25. Triton X (Sigma Aldrich)
26. Blocking Aid Solution (Invitrogen)
27. Alexa 546 (Invitrogen)
28. Alexa 488 (Invitrogen)
29. 2nd antibody in Blocking Aid (Invitrogen)
30. Hoechst 33342 (Invitrogen)
31. Nail Polish (essence color and go)
32. Trizol LS Reagent (Invitrogen)
33. Chloroform (Sigma Aldrich)
34. pure Link RNA Mini Kit (Invitrogen)
35. 2 - mercaptoethanol (Sigma Aldrich)
36. DNAse I amplification grade (Invitrogen)
37. Superscript II reverse transcriptase (Invitrogen)
38. Agarose (Invitrogen)
39. Ladders (Invitrogen)
40. DNA Loading Dye (Ambion)

Methods

Cell Culture

Plasmodium falciparum lab strain 3D7 was cultured in vitro according to the protocol by Trager and Jensen (1976). Briefly, P. falciparum was cultured in Human O+ erythrocytes at 5% haematocrit in culture media containing RPMI 1640 (Invitrogen) supplemented with L glutamine and HEPES, 10% Albumax II (Invitrogen), 2% Human AB+ serum, 1% Hypoxanthine (ccpro) and 0.05% Gentamicin (Invitrogen) in 92% N2, 5% CO2, 3% O2. Parasite growth was then followed by examination and counting parasitemia using giemsa stained thin smears.

In vitro drug sensitivity assay and classical delayed death assay

Stock drug solutions of Clindamycin hydrochloride (Sigma Aldrich), Chloroquine (Sigma Aldrich) and Fosmidomycin (Sigma Aldrich), were prepared in dimethyl sulfoxide (DMSO) and in water respectively. Working solutions were then prepared in
incomplete culture media containing only RPMI 1640 (Invitrogen) supplemented with L glutamine and HEPES, 1% Hypoxanthine (ccpro) and 0.05% Gentamicin (Invitrogen). One hundred microliters of incomplete culture media was then added into 96 wells culture plate. Into this, 100 µl of \( P. falciparum \) cultures synchronized with 5% D-Sorbitol (Lambros and Vanderberg 1979) and diluted to 2% hematocrit and 0.1% parasitemia was then added. Drugs were later added in a threefold dilution in quadruplicates. The cultures were then incubated at 37°C in 92% N\(_2\), 5% CO\(_2\), 3% O\(_2\) for 48, 72, 96 and 120 hours. Parasite growth was then determined by SYBR green assay (Smilkstein et al., 2004). IC50 was calculated using Excel sheets (Microsoft Office for mac 2011) (Figure 6 below, and Figure 18 under results).

To determine effects of clindamycin exposure after invasion (the so called delayed death assay) (Figure 4 below), \( P. falciparum \) cultures were tightly synchronized with D-Sorbitol (Lambros and Vanderberg 1979). Then ring stages of the parasites which were about 6 hours old post invasion, were used to initiate different cultures. These were then treated with IC99 concentrations of either of the following drugs:

- 50 nM clindamycin
- 128.7 µM Fosmidomycin

One group of the culture was was used to set up a negative control in which the parasites were only treated with DMSO. Growth and development of the cultures was then followed by examination using giemsa stained thin smears and light microscopy for two cycles.
Figure 4 Experimental set up to determine clindamycin half maximum inhibitory concentrations and effects of clindamycin exposure after invasion. Synchronised parasite cultures were treated with clindamycin at 6 hours post invasion. To assay clindamycin half maximum inhibitory concentrations parasite growth was then assayed by SYBR green assay (fluorescence detection assay) at 78, 102 and 126 hours post invasion. To assay effects of clindamycin exposure after invasion parasite growth was then determined by light microscopy at 12 hours intervals until 108 post invasion.

**In vitro Clindamycin Exposure Before Invasion**

To determine effects of clindamycin exposure before invasion (Figure 5 below; Synchronisation of drug pressure and parasite cycle); briefly, merozoites were isolated according to the protocol by Boyle et al., (2010 B) and exposed to clindamycin before invasion (Figure 5 below). This was done by first tightly synchronizing *P. falciparum* cultures with D-Sorbitol (Lambros and Vanderberg 1979) and heparin (Boyle et al., 2010 A and B). Then after 40 - 42 hours post invasion the cultures were treated with E64 (Sigma Aldrich) and incubated for 5-8 hours. The cultures were then filtered in incomplete culture media through 1.2 µm syringe filters (Sartorius). Equal portions of the merozoites filtrate were used to initiate different cultures that were treated before invasion with 50nM clindamycin. A portion of the filtrate was used to set up a negative control in which the merozoites were not
exposed to any drug treatment and a positive control in which the merozoites were initially not exposed to any drug treatment but after 6 hours post invasion the cultures were treated with 50 nM clindamycin. Growth and development of the cultures was then followed by examination using giemsa stained thin smears and light microscopy for two cycles.

Figure 5 Experimental set up to synchronise clindamycin drug pressure and parasite cycle. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. The cultures were then followed for two cycles by assaying parasites growth and development using light microscopy.

**Determination of the effect of synchronising clindamycin drug pressure and parasite cycle on the Apicoplast biogenesis**

The effect of synchronising clindamycin drug pressure and parasite cycle on the apicoplast biogenesis (Figure 6 below) was determined using immunofluorescence assay (IFA) as described by Tonkin *et al.*, (2004). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then cells were harvested from these cultures after every 12 hours for 2 cycles (Figure 6 below). They were then washed with 1X PBS and fixed using 4% Paraformaldehyde (AppliChem) and 0.75% Gluteraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at
37°C. After fixation, they were washed with 1x PBS and thin smears were made on Poly - L - Slides (Sigma Aldrich). Permeabilization was then done by treating them with 0.2% Triton X-100 (Carl Roth) in 1X PBS for 30 minutes at room temperature. They were washed with 1X PBS and blocked by treating them with Blocking Aid Solution (BAS) (Invitrogen) for 1 hour at room temperature in a humidified chamber. Anti-ACP primary antibodies diluted 1:250 in Blocking Aid Solution (Invitrogen) were then applied. The cells were then incubated for 1-2 hours in a dark humidified chamber at room temperature. They were then washed three times in 1X PBS and then secondary antibodies; Alexa Fluor 546 (Invitrogen) diluted 1:1000 in Blocking Aid Solution (Invitrogen) were applied. They were then incubated for 1 hour in a dark humidified chamber at room temperature and then washed three times in 1X PBS. Hoechst 33342 (Invitrogen) diluted 1:1000 in 1x PBS was then added and the cells were incubated for 5 minutes in a humidified dark chamber at room temperature. They were then washed three times in 1X PBS. Finally, they were mounted with Prolong Gold mounting solution (Invitrogen) and examined with a fluorescence microscope (Zeiss Observer.Z1).

Figure 6 Experimental set up to determine the effect of synchronised clindamycin drug pressure and parasite cycle on the apicoplast biogenesis in *P. falciparum*. *P. falciparum* was cultured in vitro and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Apicoplast biogenesis was then assayed using immunofluorescence assay and fluorescence microscopy for two cycles.
Rescue Of Clindamycin Arrested Schizonts With Isopentenyl Pyrophosphate

Rescue of clindamycin-arrested schizonts with isopentenyl pyrophosphate (Figure 7 below), was done according to the protocol by Yeh and DeRisi (2011). Briefly, different groups of cultures were first exposed to clindamycin before invasion as described above. They were subsequently treated by adding 200 µM of IPP (Sigma Aldrich) at different time points post invasion, namely at 48, 60, 72, 84 and 96 hours. As controls, one group of cultures exposed to clindamycin before invasion and the negative control were not treated with IPP.

![Diagram of experimental set up](image)

**Figure 7** Experimental set up to rescue clindamycin treated parasites with IPP. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Then in different groups, apicoplast’s function was complemented by providing clindamycin treated parasites with IPP at different time points (at 48, 60, 72, 78, 84 and 96 hours post invasion). As a negative control, one group was neither treated with clindamycin nor with IPP (Untreated). And as a positive control; one group was treated only with clindamycin and then only with water (Clindamycin). Parasite growth was then monitored at the beginning of the third cycle by assaying for ring stages of the parasites, which are indicators of a new cycle.
**Induction of Egress with Zaprinast**

Induction of Egress in clindamycin-arrested schizonts with zaprinast (Figure 8) was done according to the protocol of Collins *et al.*, (2013). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. They were subsequently treated by adding 100 µM of Zaprinast (Sigma Aldrich) at different times post invasion, namely: at 48, 60, 72, 84 and 95 hours. As controls, one group of cultures exposed to clindamycin before invasion and the negative control were not treated with zaprinast.

**Figure 8** Experimental set up to determine rescue of clindamycin treated parasites with zaprinast. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Then in different groups, the parasite cultures were treated with zaprinast in different groups and at different time points (48, 60, 72, 78, 84 and 96 hours post invasion). As a negative control, one group was neither treated with clindamycin nor with zaprinast. And as a positive control; one group was treated only with clindamycin and then only with DMSO. Parasite growth was then monitored at the beginning of the third cycle by assaying for ring stages of the parasites, which are indicators of a new cycle.

**Mechanical rupture of clindamycin-arrested schizonts**

Mechanical rupture of clindamycin-arrested schizonts (Figure 9 below) was done by
first synchronizing drug pressure and parasite cycle as described above. Then towards the end of the 2nd cycle merozoites were isolated as described above. These were used to initiate new cultures. Parasite growth was then monitored at the beginning of the third cycle.

**Figure 9** Experimental set up to mechanically induce egress of clindamycin arrested schizonts. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Then towards the end of the second cycle, merozoites were then isolated and used to initiate new cultures. Growth of the cultures was then determined by assaying for ring stages of the parasites at the beginning of the third cycle, which are indicators of new cycle.

**Determination Of The Effects Of Clindamycin-Induced Inhibition Of The Apicoplast Biogenesis On Pfsub1 Expression**

The effect of clindamycin-induced inhibition of the apicoplast biogenesis on PfSUB1 expression (Figure 10 below), was determined using reverse transcriptase PCR according to the protocol by Sanyal *et al.*, (2013). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then schizonts
were harvested by magnetic purification at the end of the second cycle. From these samples, total RNA was then isolated using pure Link RNA Mini Kit (Ambion) according to the manufacturers instructions. Potential DNA contaminants were eliminated by treating the samples with DNase (Invitrogen) according to the manufacturers protocol. The product from this reaction was used as a template for complementary DNA (cDNA) synthesis. This was performed using superscript II reverse transcriptase (Invitrogen) according to the manufacturers instructions. Finally, lack of DNA contamination was confirmed by PCR (Table 2 below) with the primer pair 1 (Table 1 below) followed by DNA quantification using agarose gel electrophoresis (Supplementary figure 1). Real time PCR was then performed under the following conditions (Table 2) with the primer pairs 2 and 3 (Table 1).

![Diagram of experiment setup](image)

**Figure 10** Experimental set up to determine the effects of clindamycin-induced inhibition of the apicoplast biogenesis on *PfSUB1* expression. *P. falciparum* was cultured in vitro and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Samples were harvested towards the end of the 2nd cycle. Then gene expression analysis was done using real time PCR.
Table 1 Primers

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<th>Gene</th>
<th>Sequence (5’ to 3’ orientation)</th>
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<tr>
<td>1. Erythrocyte binding antigen-165</td>
<td>AAAGCTGAATCTTGCCCGTT</td>
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<tr>
<td></td>
<td>GCAGGGAAAGGTCTTTCAAG</td>
</tr>
<tr>
<td>2. Arginine-tRNA ligase (Pfatl)</td>
<td>AAGAGATGCATGTTGTC</td>
</tr>
<tr>
<td></td>
<td>GTACCCAATCACCTA</td>
</tr>
<tr>
<td>3. Subtilisin-like protease 1 (Pfsub1)</td>
<td>TAAAGGGAATTTTGCTCAAC</td>
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<tr>
<td></td>
<td>ATTTTCGACTCTAACATACT</td>
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Table 2 PCR conditions used to confirm lack of DNA contamination

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<th>Temp (°C)</th>
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Table 3 RT PCR conditions

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Determination of The Effects Of Clindamycin-Induced Inhibition Of The Apicoplast Biogenesis On Secretion Of Pfsub1

The effect of clindamycin-induced inhibition of the apicoplast biogenesis on secretion of Pfsub1 (Figure 11 below), was determined using immunofluorescence assay (IFA) according to the protocol by Collins et al., (2013). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then cells were harvested from these cultures towards the end of the second cycle. They were then washed with 1X PBS and fixed using 4% Paraformaldehyde (Agar Scientific) and 0.0075% Gluteraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at room
temperature. After fixation, they were washed with 1x PBS and thin smears were made on Poly - L - Slides (Sigma Aldrich). Permeabilization was done by treating them with 0.1% Triton X-100 (Carl Roth) in 1X PBS for 10 minutes at room temperature. They were washed with 1X PBS and blocked by treating them with Bovine Serum Albumin (BSA) (Roth) for 1 hour at room temperature in a humidified chamber. Anti-PfSUB1 as primary antibodies diluted 1:250 in PBS (Sigma Aldrich) /0.5% (v/v) Tween 20 (Sigma Aldrich) /1% (w/v) BSA (Roth) were then applied. The cells were then incubated for 1-2 hours in a dark humidified chamber at room temperature. They were then washed three times in 1X PBS and then the secondary antibodies (Alexa Flour 548 (Invitrogen)) diluted 1:1000 in PBS (Sigma Aldrich) /0.5% (v/v) Tween 20 (Sigma Aldrich) /1% (w/v) BSA (Roth) were then applied. The cells were then incubated for 1 hour in a dark humidified chamber at room temperature and then washed three times with 1X PBS. Hoechst 33342 (Invitrogen) diluted 1:1000 in 1x PBS was then added and the cells were incubated for 5 minutes in a dark humidified chamber at room temperature. They were then washed three times with 1X PBS. Finally, they were mounted with Pro-long Gold mounting solution (Invitrogen) and examined on a fluorescence microscope (Zeiss Observer.Z1).
Figure 11 Experimental set up to determine the effect of clindamycin induced inhibition of the apicoplast function on PFSUB1 secretion in \textit{P. falciparum}. \textit{P. falciparum} was cultured in vitro and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. PFSUB1 localisation was then assayed using immunofluorescence assay and fluorescence microscopy for two cycles.

**Determination of The Effects Of Clindamycin-Induced Inhibition Of The Apicoplast Biogenesis On Ca$^{2+}$ Signalling**

The effects of clindamycin-induced inhibition of the apicoplast biogenesis on Ca$^{2+}$ signalling (Figure 12 below), was determined according to the protocol by Agarwal \textit{et al.}, (2013). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Towards the end of the second cycle the cultures were treated with 0.1 µM Fluo - 4AM (Invitrogen) and incubated for 20 minutes at 37° C. They were then washed 4 times by changing the media and centrifugation at 4000 X g for 3 minutes. The pellets were then re suspended with 3 ml incomplete culture
media and then the mean fluorescence intensity (MFI) was acquired using a FACS machine under the following settings: FSC - 66, SSC - 192, FITC - 418 and 50,000 events.

Figure 12 Experimental set up to determine effects of clindamycin induced inhibition of the apicoplast function on calcium signalling. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Levels of intracellular Ca²⁺ were then assayed towards the end of the second cycle using flow cytometry.

**Determination of The Effect Of Clindamycin-Induced Inhibition Of The Apicoplast Biogenesis On Phosphoinositides Metabolism**

The effect of clindamycin-induced inhibition of the apicoplast biogenesis on phosphoinositides metabolism, was done as summarized in Figure 13 below. Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then towards the end of the second cycle the samples were processed and phosphoinositide was measured using phosphatidylinositol 3
phosphate (PI(3)P) mass elisa kit (echelon biosciences) and phosphatidylinositol 4 phosphate (PI(4)P) mass strip kit (echelon biosciences) according to the manufacturers protocols.

Figure 13 Experimental set up to determine effects of clindamycin induced inhibition of the apicoplast function on phosphoinositide metabolism. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Levels of phosphoinositides; phosphatidylinositol 3 phosphate (PI3P) and phosphatidylinositol 4 phosphate (PI4P), were then assayed towards the end of the second cycle using flow cytometry and mass strips or mass elisa kits respectively.

**Determination of ADEPS *in vitro* activity against Plasmodium falciparum**

*In vitro* activity of ADEPS against *Plasmodium falciparum* was determined as summarized in Figure 14 below. Briefly, *P. falciparum* was cultured *in vitro*, tightly synchronized with D-Sorbitol according to the protocols by Lambros and Vanderberg (1979) and heparin by Boyle *et al.*, (2010 A and B). Then after 40 - 42 hours post
invasion the cultures were treated with E64 (Sigma Aldrich) and incubated for 5-8 hours. The cultures were then filtered in incomplete culture medium through 1.2 µm syringe filters (Sartorius) according to the protocol by Boyle et al., (2010 B). Equal portions of the merozoites filtrate were used to initiate different cultures that were treated before invasion with either of the following drugs:

- ADEP 1 at 10 µg/ml and 30µg/ml
- ADEP 4 at 10 µg/ml and 30µg/ml

A portion of the filtrate was utilised to set up a negative control in which the merozoites were not exposed to any drug treatment. Growth and development of the cultures was then followed by examination using giemsa stained thin smears and light microscopy for two cycles.

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**Figure 14** Experimental set up to determine *in vitro* activity of ADEPS against *P. falciparum*. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to ADEP1 or 4 before invasion. The cultures were then followed for two cycles by assaying parasites growth and development using light microscopy.
Determination of the effects of ADEP4 on apicoplast biogenesis

The effect of ADEP4 on apicoplast biogenesis, was determined (Figure 15 below) using immunofluorescence assay (IFA) as described by Tonkin et al., (2004). Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then cells were harvested from these cultures towards the end of the first and second cycles (Figure 15 below). They were then washed with 1X PBS and fixed using 4% Paraformaldehyde (AppliChem) and 0.75% Glutaraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at 37°C. After fixation, the cells were washed with 1x PBS and thin smears were made on Poly - L - Slides (Sigma Aldrich). Permeabilization was done by treating them with 0.2% Triton X-100 (Carl Roth) in 1X PBS for 30 minutes at room temperature. They were washed with 1X PBS and blocked by treating them with Blocking Aid Solution (BAS) (Invitrogen) for 1 hour at room temperature in a humidified chamber. Anti-ACP primary antibodies diluted 1:250 in Blocking Aid Solution (Invitrogen) were then applied. The cells were then incubated for 1-2 hours in a dark humidified chamber at room temperature. They were then washed three times with 1X PBS and then secondary antibodies; Alexa Fluor 546 (Invitrogen), diluted 1:1000 in Blocking Aid Solution (Invitrogen) were applied. The cells were then incubated for 1 hour in a dark humidified chamber at room temperature and then washed three times in 1X PBS. Hoechst 33342 (Invitrogen) diluted 1:1000 with 1x PBS was then added and they were incubated for 5 minutes in a dark humidified chamber at room temperature. They were then washed three times with 1X PBS. Finally, they were mounted with Pro-long Gold mounting solution (Invitrogen) and examined with a fluorescence microscope (Zeiss Observer.Z1).
Figure 15 Experimental set up to determine effects of ADEP4 on apicoplast biogenesis in *P. falciparum*. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to ADEPS 1 or 4 before invasion. Apicoplast biogenesis was then assayed using immunofluorescence assay and fluorescence microscopy towards the end of the first and second cycle.

**Determination of the effects of ADEP4 on apicoplast function**

To determine effect of ADEP4 on apicoplast function (Figure 16 below); different groups of cultures were first exposed to ADEPS before invasion as described above. They were then treated by adding 200 µM of IPP (Sigma Aldrich) at 48 hours post invasion. As controls, one group of cultures exposed to ADEPS before invasion and the negative control were not treated with IPP.
Chemical Knock - Out of the apicoplast

Chemical knock-out of the apicoplast (Figure 17 below) was performed according to the protocol by Yeh and DeRisi (2011). Briefly, merozoites were isolated as described above, they were then treated with 50nM clindamycin before invasion and used to initiate cultures. Starting at 48 hours post invasion the cultures were treated by adding 200 µM of IPP (Sigma Aldrich). Then after 48 hours, the culture media was replaced with fresh media and IPP. As controls, one group of cultures exposed to clindamycin before invasion and the negative control were not treated with IPP.
Figure 17 Experimental set up to determine whether the apicoplast is the target for ADEPS. *P. falciparum* was cultured *in vitro* and tightly synchronised. Merozoites were then isolated and exposed to clindamycin before invasion. Starting at 48 hours post invasion, the cultures were then maintained under continuous exogenous IPP provision for several cycles. The parasites were subsequently treated with ADEP4, maintained under continuous exogenous IPP provision and followed for two cycles by assaying parasites growth and development using light microscopy.

**Data Analysis**

Graphs for medians, means and standard deviations of treatment groups were generated using excel (Microsoft office 2010 for macs). Data analysis was performed using Stata® (Version 12.1 Statacorp USA). This was done by comparing the medians or means from the different treatment groups using the Kruskal-wallis rank test and a P value of 0.05.
RESULTS

Part 1

Clindamycin causes an egress defect of *P. falciparum*

In order to solve the puzzle regarding the discrepancy in the response of *P. falciparum* to clindamycin during its liver and blood stages, this study mimicked the experimental set up in the liver stage studies by Friesen *et al.*, (2010). To begin with, *P. falciparum* blood stage was cultured *in vitro*. Then its sensitivity to clindamycin was determined by growth inhibitory assay on exposure post invasion (Figure 4). As compared to chloroquine and fosmidomycin, the parasite sensitivity to clindamycin increased with time (Figure 18 and 19 below). This is a classical phenotype termed delayed death (Surolia *et al.*, 2004; Burkhardt *et al.*, 2007; Dahl and Rosenthal 2007; Goodman *et al.*, 2007). This confirmed that the drug that was utilized was working.

![Graph showing IC50 values for chloroquine, fosmidomycin, and clindamycin](image)

**Figure 18** Half maximum inhibitory concentration values (IC50) of clindamycin. *P. falciparum* was treated with clindamycin 6 hours post invasion. Parasite growth was then determined after 72, 96 and 120 hours post invasion. Chloroquine and fosmidomycin were used as control drugs. Note that the parasites only become sensitive to clindamycin after two cycles as opposed to one cycle under chloroquine or fosmidomycin. For clindamycin the IC50 values were 15, 48 nM/L and 113,647 nM/L at 126, 102 and 78 hours post-invasion respectively. For chloroquine the IC50 values were 7,8 and 9 nM/L at 126, 102 and 78 hours post-invasion respectively. For fosmidomycin the IC50 values were 716, 524 and 2249 nM/L at 126, 102 and 78 hours post-invasion respectively.
Figure 19 Growth inhibitions of *P. falciparum* treated with clindamycin 6 hours post invasion. Note the arrest of clindamycin treated parasites during the second cycle as opposed to the first cycle in fosmidomycin treated parasites. Error bars represent standard deviation of the mean.

In order to study the effects of synchronized antibiotic drug pressure and parasite cycle during the blood stage (Figure 5 under materials and methods section), *P. falciparum* was cultured *in vitro* in human erythrocytes. These cultures were then tightly synchronised using D-Sorbitol, heparin and magnetic columns as described in materials and methods. From a population of schizonts only, merozoites were then isolated and exposed to clindamycin before invasion. Parasite growth was subsequently monitored for 2 cycles by light microscopy.

During the first cycle, there was no growth inhibition in both the treated and untreated parasite cultures (Figure 20). In both cases, parasites managed to egress from the erythrocytes, invaded uninfected erythrocytes, initiating a second cycle. During this second cycle, they both transformed into rings, trophozoites and schizonts. The untreated parasites were yet again able to egress and invade uninfected erythrocytes. However, the treated parasites were arrested towards the end of the second cycle as segmented schizonts (Figure 21). This indicated that clindamycin induces an egress defect.
Figure 20 Growth inhibition of *P. falciparum* on treatment with clindamycin before invasion. Note the arrest during the second cycle of parasites treated with clindamycin before invasion (Test) or post invasion (Positive control). Error bars represent standard deviation of the median.

Figure 21 Thin smears of *P. falciparum in vitro* cultures during the second cycle post invasion. The bottom row represents parasites treated with clindamycin and the top row is the control. Note the arrested schizonts towards the end of the second cycle and beginning of the third cycle in clindamycin treated parasites (Clindamycin Hour 84 - 96).
Inhibition of egress correlates with inhibition of the apicoplast biogenesis.

The antimalarial effect of clindamycin is based on the inhibition of the apicoplast biogenesis and inheritance (Camps et al., 2002; Goodman et al., 2007; Friesen et al., 2010). Therefore in order to determine the fate of the apicoplast under synchronized drug exposure and parasite cycle, the biogenesis of the apicoplast was assayed by immunofluorescence assay and fluorescence microscopy (Figure 6 under materials and methods section).

During the first cycle, in both the treated and untreated cultures, the organelle grew, branched and got segregated into daughter cells (Figure 22 and 23), which were able to egress and invade uninfected erythrocytes. Towards the end of the second cycle, the organelle in the control was able to grow, branch and get segregated to daughter cells (Figure 24 and 25). These were then able to egress and invaded uninfected erythrocytes. In contrast, the clindamycin-arrested schizonts displayed a stunted apicoplast that was not able to grow, branch and segregate (Figure 24 and 25). This indicated that the inhibition of the apicoplast biogenesis and function preceded the inhibition of egress.
Figure 22 Effects of clindamycin on the apicoplast biogenesis in *P. falciparum* during the first half of the first cycle. Shown here are immunofluorescence assay pictures of the apicoplast of *P. falciparum* treated with clindamycin before (Test) or 6 hours after invasion (Positive control) as compared to untreated cultures (Negative control). In the first half of the cycle (Hour 12-21), there was no inhibition of apicoplast biogenesis in the treated and untreated cultures. **Key:** DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 546 and Hoeschst.
Figure 23 Effects of clindamycin on the apicoplast biogenesis in *P. falciparum* during the second half of the first cycle. Shown here are immunofluorescence assay pictures of the apicoplast of *P. falciparum* treated with clindamycin before (Test) or 6 hours after invasion (Positive control) as compared to untreated cultures (Negative control). In the second half of the first cycle (Hour 36-42), there was again no inhibition of apicoplast biogenesis in the treated and untreated cultures. **Key:** DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 546 and Hoeschst.
Figure 24 Effects of clindamycin on the apicoplast biogenesis in *P. falciparum* during the first half of the second cycle. Shown here are immunofluorescence assay pictures of the apicoplast of *P. falciparum* treated with clindamycin before (Test) or 6 hours after invasion (Positive control) as compared to untreated cultures (Negative control). In the first half of the second cycle (Hour 60-66), there was again no inhibition of apicoplast biogenesis in the treated and untreated cultures. **Key:** DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 546 and Hoeschst.
Figure 25 Effects of clindamycin on the apicoplast biogenesis in *P. falciparum* during the second half of the second cycle. Shown here are immunofluorescence assay pictures of the apicoplast of *P. falciparum* treated with clindamycin before (Test) or 6 hours after invasion (Positive control) as compared to untreated cultures (Negative control). Towards the end of the second cycle (Hour 84-90), there was again no inhibition of apicoplast biogenesis in the untreated cultures, but in the treated cultures (before or after invasion) there was inhibition of apicoplast biogenesis. **Key**: DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 546 and Hoeschst.
Chemical complementation of the apicoplast function reverses egress defects

Antibiotic treated parasites can be rescued by provision of exogenous IPP (Ye and DeRisi 2011). Surprisingly, full rescue, however, was only achieved when IPP was supplemented at the beginning of the second cycle, i.e. 48 hours pre-egress (Ye and DeRisi 2011). To circumvent the clindamycin induced inhibition of the apicoplast’s biogenesis and function (Figures 22 - 25) and inhibition of the parasite egress (Figure 21), clindamycin-treated parasites were supplemented with IPP at 12 hours intervals in different groups starting at 48 hours post-invasion (Figure 7 under materials and methods section).

Because the function of the apicoplast during the blood stage is to produce IPP, therefore, ideally rescue with IPP should have been possible for all the tested time points. However, the arrested schizonts were able to transform into rings but in a proportion dependent on the time point of IPP provision (Figure 26). Such that only IPP treatment done at the time points away from egress (Hour 48, 60, 72 and 78) was able to rescue the parasites. But IPP treatment done at the time points close to egress (Hours 84) did not rescue the parasites. This indicated that the egress inhibition (Figure 21 above) is due to the clindamycin-induced shortage of IPP and that a factor or process that is needed to enable the parasites to egress depends on IPP. This factor or process starts to function early during the cycle. Therefore supplementation with IPP needs to be done early in the cycle as well.
Figure 26 IPP rescue of clindamycin treated parasites at different time points post invasion. Only provision of IPP for the time points close to invasion was able to rescue the parasites (TR H48, TR H60, TR H72 and TR H78) but time points close to egress (TR84 and TR96) did not rescue the parasites. Key: TR48, TR60, TR72, TR78, TR84 and TR96: Test sample treated with IPP at 48, 60, 72, 78, 84 and 96 hours post invasion respectively. Error bars represent standard deviation of the median.

Zaprinast induces egress of Clindamycin-arrested schizonts

Since IPP provision could not directly rescue the egress defects, chemical and mechanical means were applied to induce egress. To start with, the chemical means were used (Figure 8 above, under materials and methods section). Egress of *P. falciparum* involves the activation of Protein Kinase G (PKG) by a natural egress signal (Collins *et al.*, 2013) which can be mimicked by zaprinast. PKG, in-turn activates egress of *P. falciparum* (Collins *et al.*, 2013). Therefore, to induce egress of the clindamycin-arrested schizonts, they were treated with zaprinast at 12 hours intervals in different groups starting at 48 hours post-invasion.

As an artificial trigger of egress, zaprinast (Collins *et al*, 2013), would be expected to result in inducing rupturing of schizonts. In cultures not treated with clindamycin, according to light microscopy, zaprinast indeed induced premature egress (data not
shown) and this resulted in successful invasion (Figure 27). However, as compared to the untreated parasites, in clindamycin treated parasites zaprinast rescue did not work as expected. It induced an unusual early rescue, where, only parasites treated with zaprinast; immediately, after invasion were able to progress to a 3rd cycle (Figure 28). And according to light microscopy, clindamycin arrested schizonts did not rupture when treated with zaprinast towards egress (data not shown). This means that; only early treatment with zaprinast, can reverse clindamycin effects. And since this observation is reminiscent of; only early IPP provision also, being able to rescue the parasites (Figure 26). This suggests that; egress machinery starts getting stimulated or assembled at the beginning of the cycle.

Figure 27 Effect of zaprinast on *Plasmodium falciparum*. As compared to the control, culture treated with zaprinast at 43 hours post invasion i.e. towards the end of the second cycle, were still able to progress to a third cycle.
Mechanically released merozoites from clindamycin-arrested schizonts are non-invasive

The ability of merozoites to egress is a prerequisite to invasion (Collins et al., 2013). PKG and calcium dependent protein kinase 5 (CDPK5) regulate the egress pathway (Dvorin et al., 2010). In addition PKG also regulates invasion (Alam et al., 2015). Mechanical rupture of arrested schizonts due to inhibition of these proteins has been used to investigate the egress pathway (Dvorin et al., 2010). Therefore, failure to induce egress by chemical means led to the attempt to circumvent the clindamycin-induced block of the parasite egress by mechanical rupture of the arrested schizonts (Figure 9 above, under materials and methods section). These mechanically induced egress produced merozoites with normal morphology.
(data not shown), indicating that the arrested merozoites are ready to egress but unable to. Therefore confirming the observed egress defect phenotype (Figure 21 above). However, these mechanically-released-merozoites were unable to invade (Figure 29) indicating that they do not only have egress defects but also invasion defects. This is a phenotype similar to treatment with PKG inhibitors during egress (Dvorin et al., 2010) and invasion (Alam et al., 2015). Nonetheless, it is different to CDPK5 knock down (Dvorin et al., 2010). This suggested clindamycin inhibition of the apicoplast biogenesis and function inhibits the egress pathway.

![Figure 29 Invasion assay at 12 hours post invasion.](image)

**Figure 29 Invasion assay at 12 hours post invasion.** As compared to the control, merozoites released by mechanical rupture of clindamycin arrested schizonts were not able to invade erythrocytes. P values represent Kruskal wallis rank test of the medians and error bars represents standard deviation of the median.

**Clindamycin-induced inhibition of the apicoplast does not inhibits expression of PfSUB1**

During egress, PKG functions to regulate the secretion of PfSUB1 (Collins et al.,
2013) from exonemes into the parasitophorous vacuole (Yeoh et al., 2007). PfSUB1 is a serine protease which links egress and invasion (Koussis et al. 2009). It mediates the activation of proteins required for egress, namely: serine like antigens (SERAs) (Collins et al., 2013) and *P. falciparum* perforin like proteins (PfPLPs) (Garg et al., 2013), as well as proteins required for invasion, namely; merozoite surface proteins 1, 6 and 7 (MSP1, 2 and 7) (Koussis et al. 2009). Clindamycin arrested schizonts; are neither able to naturally egress (Figure 21), nor upon chemical induction by treatment with zaprinast (Figure 28 above) or upon mechanical induction (Figure 29 above). In addition the merozoites from this schizonts are unable to invade (Figure 29 above). Therefore, expression and immunolocalization of PfSUB1 was performed to determine whether these parasites are able to secret PfSUB1 from exonemes (Figures 10 and 11 above, under materials and methods section).

Firstly, expression of PfSUB1 was determined by analysing the expression of *Plasmodium falciparum* subtilisin like serine protease-1 (Pfsub1, accession number PF3D7_0507500) and Arginine-tRNA ligase (Pfatl, accession number PF3D7_1218600) as a house keeping gene (Figure 10 above, under materials and methods section). There was no significant difference between the control and treated parasites in the expression of Pfsub1 and Pfatl indicating that clindamycin arrested schizonts are indeed metabolic active (Figure 30 below).

![Figure 30 Assay of PfSUB1 expression.](image)

*Figure 30 Assay of PfSUB1 expression.* There was no significant differences in the expression of a house keeping gene (Pfatl) and a non-house keeping gene (Pfsub1) between the treated and untreated cultures. *P* values represent Kruskal wallis rank test of the medians and error bars represents standard deviation of the median.
Clindamycin-induced inhibition of the apicoplast inhibits secretion of PfSUB1

Secondly, to determine whether clindamycin arrested schizonts are able to secrete PfSUB1 from exonemes, localization of PfSUB1 was determined by immunofluorescence assay and fluorescence microscopy at the end of the second cycle (Figure 11 above, under materials and methods section).

Anti-PfSUB1 antibodies, indicated a signal ranging from absent, weak to diffuse in untreated cultures (Figure 31), a characteristic of secreted PfSUB1 (Collins et al., 2013). However, treated cultures had strong and punctate signal (Figure 32) characteristic of unsecreted PfSUB1 (Collins et al., 2013). On early treatment with IPP, a product of the apicoplast function, the appearance of PfSUB1 signal ranged from weak to diffuse (Figure 33). This confirmed the function of the apicoplast in egress. On early treatment with zaprinast, it appeared strong and punctate in some cells and in other cells it appeared weak and diffused (Figure 34). This indicated that clindamycin-induced inhibition of the apicoplast biogenesis and function inhibits the secretion of PfSUB1, explaining why the merozoites in clindamycin arrested schizonts have egress and invasion defects (Figures 21 and 29).
Figures 31 Immunofluorescence assay of PfSUB1 at the end of the second cycle in untreated parasites. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in untreated P. falciparum. In the various cells observed the appearance of PfSUB1 ranged from absent, weak to diffuse. Key: DIC: Cells, Anti-PfSUB1: anti-PfSUB1 primary antibodies stained with Alexa 588 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 548 and Hoeschst.
Figures 32 Immunofluorescence assay of PfSUB1 at the end of the second cycle in clindamycin treated parasites. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in P. falciparum treated with clindamycin before invasion. In the various cells observed the appearance of PfSUB1 was mostly strong and punctuated. **Key:** DIC: Cells, Anti-PfSUB1: anti-PfSUB1 primary antibodies stained with Alexa 588 secondary antibodies; DNA: Nucleus stained with Hoeschst; Merge: Combination of Alexa 548 and Hoeschst.
Figures 33 Immunofluorescence assay of PfSUB1 at the end of the second cycle in clindamycin treated parasites and rescued with IPP. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* treated with clindamycin before invasion and then with IPP at 48 hours post invasion. The appearance of PfSUB1 ranged from weak to diffuse. **Key:** DIC: Cells, Anti-PfSUB1: anti-PfSUB1 primary antibodies stained with Alexa 588 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 548 and Hoeschst.
Figures 34 Immunofluorescence assay of PfSUB1 at the end of the second cycle in clindamycin treated parasites and rescued with zaprinast. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* treated with clindamycin before invasion and then with zaprinast at 48 hours post invasion. The appearance of PfSUB1 was strong and punctate in some cells and in others it ranged from absent, weak to diffuse. **Key:** DIC: Cells, Anti-PfSUB1: anti-PfSUB1 primary antibodies stained with Alexa 588 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 548 and Hoeschst.
Clindamycin-induced inhibition of the apicoplast does not inhibit calcium signaling and phosphoinositide metabolism

PKG functions to regulate the secretion of PfSUB1 (Collins et al., 2013) from exonemes into the parasitophorous vacuole (Yeoh et al., 2007) by stimulating an increase in intracellular Ca\(^{2+}\) (Brochet et al., 2014). Increase in intracellular Ca\(^{2+}\) is required for egress of P. falciparum (Agarwal et al., 2013; Collins et al., 2013; Glushakova et al., 2013), this is because it functions to induce secretion of PfSUB1 (Agarwal et al., 2013). Therefore in order to understand how clindamycin induced inhibition of the apicoplast biogenesis and functions (Figures 21 – 26 above) inhibits PfSUB1 secretion (Figures 31 – 34 above), calcium signalling was determined under clindamycin treatment. This was done by assaying the levels of intracellular Ca\(^{2+}\), towards the end of the second cycle (Figure 12 above, under materials and methods section).

There was no significant difference in the levels of intracellular calcium ions in clindamycin treated parasites as compared to untreated parasites (Figures 35). Early rescue with IPP or zaprinast did not significantly change these levels (Figures 35). This indicated that clindamycin-induced inhibition of PfSUB1 secretion is not through the inhibition of calcium signalling.
Figures 35 Levels of free intracellular calcium ion measured at the end of the second cycle. There was no significant difference in the levels of free intracellular calcium ions in clindamycin treated parasites (Treated) as compared to untreated parasites. Early rescue with IPP (Treated+ipp) did not significantly change these levels. Even though with a borderline significant P value, early rescue with zaprinast (Treated+zap H48) appeared to increase free intracellular calcium ions but not when applied towards the end of the cycle (Untreated+zap H88). Key: ipp; isopentenyl pyrophosphate, zap; Zaprinast, H48; 48 hours post invasion, H88; 88 hours post invasion. P values represents Kruskal wallis rank test of the medians and error bars represents standard deviation of the median.

Clindamycin-induced inhibition of the apicoplast does not inhibit phosphoinositide metabolism

PKG functions to regulate the secretion of PfSUB1 (Collins et al., 2013) from exonemes into the parasitophorous vacuole (Yeoh et al., 2007), by regulating the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PI4,5BP) to increase intracellular Ca^{2+} (Brochet et al., 2014). Increase in intracellular Ca^{2+} is required for egress of *P. falciparum* (Agarwal et al., 2013; Collins et al., 2013; Glushakova et al., 2013). However, clindamycin-induced inhibition of PfSUB1 secretion and consequently the egress defects (Figure 21 above), is not through the inhibition of calcium signalling (Figure 35 above). Therefore in order to understand how clindamycin induced inhibition of the apicoplast biogenesis and functions (Figures 21 – 26 above), inhibits
PfSUB1 secretion (Figures 31 – 34 above), phosphoinositide metabolism was determined under clindamycin treatment. This was done by assaying the levels of phosphatidylinositol 3 phosphate (PI3P) and phosphatidylinositol 4 phosphate (PI4P) towards the end of the second cycle (Figure 13 above, under materials and methods section).

There was no significant difference in the levels of phosphoinositides in clindamycin treated parasites as compared to untreated parasites (Figures 36 and 37). In the case of PI4P, early rescue with IPP or zaprinast did not significantly change these levels (Figure 37). This indicated that clindamycin-induced inhibition of PfSUB1 secretion is not through the inhibition of phosphoinositide metabolism.

**Figures 36** Levels of phosphatidylinositol 3 phosphate (PI3P) measured at the end of the second cycle. There was no significant difference in the levels of PI3P in clindamycin treated parasites (treated) as compared to untreated. P values represent Kruskal wallis rank test of the medians and error bars represent standard deviation of the median.
Figures 37 Levels of phosphatidylinositol 4 phosphate (PI4P) measured at the end of the second cycle. There was no significant difference in the levels of PI4P in clindamycin treated parasites (Treated) as compared to untreated parasites. Early rescue with IPP (Treated+IPP) or zaprinast (Treated+Zaprinast) did not significantly change these levels. P values represent Kruskal wallis rank test of the medians and error bars represent standard deviation of the median.
Part 2

ADEP 4 slows down the cell cycle of *Plasmodium falciparum*

ADEPS are new type of antibacterial drugs (Brötz - Oesterhelt et al., 2005; Sass et al., 2011). Therefore, to determine whether they can act as antimalarial drugs, *P. falciparum* was exposed *in vitro* to different types of ADEPS: ADEP 1 and 4 (Figure 14 above, under materials and methods section).

ADEP 1, demonstrated two different effects: As compared to untreated parasites, at high concentrations ADEP 1 inhibited parasite growth within 72 hours (Figure 38). But, at low concentrations, ADEP 1 did not show any activity against *P. falciparum* (Figure 38). ADEP 4, also demonstrated two different effects. However, as compared to untreated parasites, at high concentrations it inhibited parasite growth within the first 24 hours (Figure 39). But, at low concentrations, it induced slow parasite growth (Figures 39 and 40). Indicating that; ADEP4 was more active against *P. falciparum* than ADEP1.

![Figure 38 Growth inhibition assay of ADEP1 on P. falciparum.](image)

ADEP1 at 10 ug/ml (ADEP1-10) did not inhibit parasite growth while ADEP1 at 30 ug/ml (ADEP1-30), inhibited parasite growth around 72 hours during the second cycle. **Key:** H24, H48, H72 and H96 means 24, 48, 72 and 96 hours post invasion respectively. Error bars represent standard deviation of the median.
Figure 39 Growth inhibition assay of ADEP4 on *P. falciparum*. ADEP4 at 10 µg/ml (ADEP4-10) induced slow parasite growth while ADEP4 at 30 µg/ml (ADEP4-30), inhibited parasite growth within 24 hours during the first cycle. **Key:** H24, H48, H72 and H96 means 24, 48, 72 and 96 hours post invasion respectively. Error bars represent standard deviation of the median.

![Graph showing growth inhibition assay](image)

Figure 40 Response of *P. falciparum* to treatment with ADEP 4 at 10 µg/ml. Untreated merozoites were able to invade erythrocytes, transformed into rings, trophozoites (Untreated Hour 24) and then into schizonts (Untreated Hour 48). This in turn gave rise to merozoites that were able to invade uninfected erythrocytes and initiate a second cycle during which they again transformed into rings (Untreated Hour 48), trophozoites (Untreated Hour 72) and then into schizonts which later formed merozoites that started a new cycle (Untreated Hour 96). Merozoites treated with ADEP 4 at 10 µg/ml were able to form rings but became arrested (ADEP4 Hour 24) and transformed very late in the cycle into trophozoites (ADEP4 Hour 42), which remained arrested as trophozoites (ADEP4 Hour 48). This in turn gave rise to merozoites that were able to invade erythrocytes and initiate a second cycle very late (ADEP4 Hour 72), at a time point, where the untreated parasites are already in the middle of their second cycle (Untreated Hour 72). They finally transform into trophozoites very late in the cycle (ADEP4 Hour 96).
**ADEP4 induces filamentation of the apicoplast**

The apicoplast is bacterial in nature (Wilson *et al.*, 1996) and since it cannot be formed de novo it has to be inherited from the mother cell to the daughter cell. In order for this to happen it first undergoes biogenesis, whereby it starts as a tiny structure that grows, branches and eventually gets segregated to the daughter cells, which later egress and invade uninfected erythocytes (Waller and McFadden 2005). Since ADEP4 has inhibitory effects against *P. falciparum* (Figures 38 and 39), therefore in order to determine whether the apicoplast was its target, its biogenesis was studied using immunofluorescence assay at the end of the first and second cycle under treatment with ADEP 1 and 4 at 10 µg/ml (Figure 15 above, under materials and methods section).

The apicoplast biogenesis under ADEP1 was not affected both during the end of the first and second cycle (Figures 41 A and B). However, under ADEP4 treatment, at the time point, where it should have undergone segregation i.e. at the end of the first and second cycle, it remained still branched (Figures 41 A and B). This morphology is typical of trophozoites, confirming that ADEP4 delays the parasite cycle during the blood stage and it does this, through inhibition of the apicoplast biogenesis.
Figure 41 Effects of ADEPS on the biogenesis of the apicoplast during the first (A) and second cycle (B). As compared to untreated parasites, ADEP1 had no effect on the apicoplast biogenesis. ADEP4 however inhibited the biogenesis of the apicoplast. Key: DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies; DNA: Nucleus stained with Hoeschst; Merge: Combination of Alexa 548 and Hoeschst.
Chemical complementation with IPP does not rescue ADEP4 treated parasites

The apicoplast functions during the erythrocytic schizogony to provide isopentenyl pyrophosphate (IPP) (Jomaa et al., 1999; Ye and DeRisi 2011). By inhibiting the biogenesis of the apicoplast (Figure 41 above) how is ADEP4 affecting parasite cycle during blood stage? Therefore, to test whether this effect of ADEP4 is through the inhibition of the apicoplast function, *P. falciparum* was treated with ADEP4 and maintained under continuous exogenous IPP provision (Figure 16 above, under materials and methods section).

 Provision of IPP did not rescue the parasites from ADEP4 treatment at 10 µg/ml and 30 µg/ml (Figure 42). At 30 µg/ml parasite growth was still inhibited within the first cycle. At 10 µg/ml, parasite transformation was not inhibited within the first cycle. Actually, parasite cycle was even much more prolonged (Figure 43 below) as compared to treatment with 10 µg/ml only (Figures 39 and 40 above). This indicates that, ADEP4 may not be only affecting the function of the apicoplast in IPP production, but ADEP4 has other targets besides inhibiting the apicoplast biogenesis and function, or perhaps the apicoplast has other unknown functions.

Figure 42 Growth inhibition assay of ADEP4 on *P. falciparum* under IPP provision. Parasites supplemented with IPP were still affected by ADEP4 at 10 µg/ml (ADEP4-10 IPP) and 30 µg/ml (ADEP4-30 IPP) as compared to control treated with DMSO and IPP. Key: H24, H48, H72 and H96 means 24, 48, 72 and 96 hours post invasion respectively. Error bars represent standard deviation of the median.
Figure 43 Response of *P. falciparum* to treatment with ADEP 4 at 10 µg/ml under exogenous provision with IPP. Untreated merozoites were able to invade erythrocytes, transformed into rings, trophozoites (Untreated Hour 24) and then into schizonts (Untreated Hour 48). This in turn gave rise to merozoites that were able to invade uninfected erythrocytes and initiate a second cycle during which they again transformed into rings (Untreated Hour 48), trophozoites (Untreated Hour 72) and then into schizonts which later formed merozoites that started a new cycle (Untreated Hour 96). Merozoites treated with ADEP 4 at 10 µg/ml and maintained under exogenous provision with IPP were able to form rings (ADEP4+IPP Hour 24) but became arrested and transformed very late in the cycle into trophozoites (ADEP4+IPP Hour 42), which remained arrested (ADEP4+IPP Hour 48 and Hour 72). These in turn gave rise to merozoites very late, which were able to invade uninfected erythrocytes and initiate a second cycle (ADEP4+IPP Hour 96) at a time point when untreated parasites were already into their 3rd cycle (Untreated Hour 96).

**Chemical knockout of the apicoplast does not rescue ADEP4 treated parasites**

Cellular materials and organelles that cannot be synthesised denovo have to be replicated and passed on to the daughter cells. And because ADEP4 is affecting the apicoplast biogenesis and its segregation, therefore to test whether this could be affecting the parasite cell cycle, the apicoplast was chemically knocked out using clindamycin. The parasites were maintained under continuous exogenous IPP provision (Figure 17 above, under materials and methods section) and then treated with ADEP4 at 10 and 30 µg/ml under continuous IPP provision.

Clindamycin induced inhibition of the apicoplast biogenesis (Figure 44) and consequently parasite growth (Figure 45). Upon early IPP provision, these parasites survived the effects of clindamycin (Figure 45). However, on treatment with ADEP4 parasite growth was still inhibited (Figure 46). This confirms that ADEP4 has other targets besides the apicoplast.
**Figure 44 Chemical knock-out of the apicoplast.** As compared to untreated parasites, in clindamycin treated parasites biogenesis of the apicoplast was inhibited (Treated). **Key:** DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies; DNA: Nucleus stained with Hoeschst; Merge: Combination of Alexa 548 and Hoeschst.

**Figure 45 Chemical rescue of clindamycin treated parasites with IPP.** As compared to untreated parasites (Control), clindamycin treated parasites were inhibited towards the end of the second cycle (Clindamycin). However, on early provision with IPP at 48 hours post invasion these parasites survived (Clindamycin + IPP). P values represent Kruskal wallis rank test of the medians and error bars represent standard deviation of the median.
Figure 46 Growth inhibition assay of ADEP4 on *P. falciparum* without the apicoplast under IPP provision. These parasites without the apicoplast but maintained under a continuous IPP supply were still affected by ADEP4 at 10 µg/ml (IPP ADEP 10) and 30 µg/ml (IPP ADEP 30) as compared to the control treated with DMSO and IPP (IPP DMSO). **Key:** H24, H48, H72 and H96 means 24, 48, 72 and 96 hours post invasion respectively. Error bars represent standard deviation of the median.
DISCUSSION

Part 1

Clindamycin induced inhibition of apicoplast biogenesis and *P. falciparum* growth

Exposure to clindamycin during the liver stage, results in the inhibition of the apicoplast biogenesis. Consequently this leads to inhibition of the parasite within the cycle of treatment (Friesen *et al.*, 2010). However, during the blood stage this effect occurs in the subsequent cycle after clindamycin treatment (Goodman *et al.*, 2007). To reconcile on these discrepancies, the first part of this study investigated the effects of synchronising clindamycin drug pressure and parasite cycle to the biogenesis of the apicoplast and growth of *P. falciparum* during blood stage (Figures 5 and 6 above, under materials and methods).

The data from this study demonstrates that, clindamycin exposure before invasion leads to the inhibition of the apicoplast biogenesis (Figure 22 - 25). Consequently, this impairs the egress machinery (Figures 31 - 34) leading to the arrest of the parasites as schizonts containing merozoites that are not able to egress (Figure 21). An overview of the egress mechanism, biogenesis, function and maintenance of the apicoplast in *P. falciparum* during blood stage, can provide an explanation for these observations.

**Egress Mechanism**

During its life cycle *P. falciparum* is restricted to live inside its host cell within a parasitophorous vacuole. In order for its life cycle to progress, it must come out of the host cell through a process called egress and then invade another one. While in the liver and blood stages, egress is utilised by merozoites to get out of the hepatocytes and erythrocytes respectively. In its life cycle in the mosquito, gametocytes utilise egress to get out of the erythrocytes ingested during a blood meal. Finally sporozoites also utilize egress to get out of the oocysts in the mosquito midgut.

During the blood stage, egress is a tightly regulated process requiring the enzyme Protein Kinase G (PKG) (Collins *et al.*, 2013). PKG functions by regulating phosphoinositide metabolism and intracellular calcium ion levels (Ca$^{2+}$) (Brochet *et al.*
al., 2014). It does this by activating the production of inositol - 1,4,5 - triphosphate (I145TP) (Brochet et al., 2014), which is an activator of Ca^{2+} release from the endoplasmic reticulum (Agarwal et al., 2013). This leads to an increase in intracellular Ca^{2+} (Agarwal et al., 2013; Collins et al., 2013; Glushakova et al., 2013), This increase in intracellular Ca^{2+} in-turn activates the exocytosis of the enzyme *P. falciparum* subtilisin-like protein 1 (PfSUB1) (Agarwal et al., 2013; Collins et al., 2013), from exonemes to the parasitophorous vacuole (Yeoh et al., 2007). In the parasitophorous vacuole, PfSUB1 in-turn mediates the activation of other proteins required for egress, namely: serine like antigens (SERAs) (Collins et al., 2013) and *P. falciparum* perforin like proteins (PfPLPs) (Garg et al., 2013). Eventually merozoites become released and then initiate another cycle.

**Biogenesis and function of the apicoplast**

During erythrocytic schizogony, in wild type parasites, there is normal apicoplast biogenesis and function (Figures 22 - 25). The organelle is therefore able to grow, branch and get segregated to merozoites. These are then able to egress, invade uninfected erythrocytes and develop normally (Figure 21). But in clindamycin treated parasites, at first, there is normal apicoplast biogenesis and function (Figures 22 - 23). The organelle is able to grow, branch and get segregated to daughter cells, which are able to egress, and then invade uninfected erythrocytes. But they begin to develop abnormally in terms of the apicoplast morphology (Figures 24 - 25) and parasite stages (Figure 21).

In regard to the parasite stages, they start as rings which transform normally to trophozoites that in-turn transform to schizonts. However, these do not egress and thereby become unable to initiate another cycle (Figure 21), the so called delayed death (Surolia et al., 2004; Burkhardt et al., 2007; Dahl and Rosenthal 2007; Goodman et al., 2007). In regard to the apicoplast, they begin with a small apicoplast that is dot like but its biogenesis becomes affected and indeed its morphology becomes abnormal (Figure 25). It does not branch and segregate like in the previous cycle or like in wild type parasites (Figures 22 – 25). Thereby not performing its biological function of IPP production (Yeh and Derisi 2011). Consequently during the second cycle, clindamycin treated parasites rely on early exogenous IPP provision to progress to a third cycle (Figure 26).
IPP is required for the prenylation of Rab proteins in order for them to perform their cellular functions (Howe et al., 2013; Alberts et al., 2008). Due to the interdependence between a functional phosphoinositide metabolism and functional Rab proteins at micro-domains (Alberts et al., 2008; Jean and Kieger 2012), clindamycin induced shortage of IPP would therefore be expected to inhibit isoprenylation of Rab proteins (Howe et al., 2013) and therefore their functions. Consequently, this would affect phosphoinositide metabolism. This shortage of IPP in turn would be expected to impair the egress machinery. This is because an inhibited phosphoinositide metabolism cannot respond to PKG induced hydrolysis of PI 4,5 BP to Inositol 1,4,5 triphosphate (I145TP) (Brochet et al., 2014). Therefore, lack of I145TP would lead to the inability to stimulate the endoplasmic reticulum to release Ca\(^{2+}\) (Agarwal et al., 2013; Brochet et al., 2014). This eventually would lead to lower intracellular Ca\(^{2+}\) which should impair the secretion of PfSUB1 from exonemes (Agarwal et al., 2013) and consequently the egress defect (Figures 21 and 28 - 34).

While this is would be expected to result from the clindamycin induced inhibition of phosphoinositide metabolism and consequently Ca\(^{2+}\) signalling. However, there is no significant difference in phosphoinositide metabolism and calcium signalling in clindamycin treated parasites as compared to the untreated parasites (Figures 35 - 37). Because early treatment with IPP and zaprinast can rescue clindamycin arrested parasites (Figures 26 and 28), their provision to clindamycin arrested schizonts would be expected to reverse the clindamycin induced effects on the inhibition of *P. falciparum*. However, IPP and zaprinast rescue had no effect on phosphoinositide metabolism (Figures 36 and 37) and calcium signalling (Figure 35). But they both reversed clindamycin induced inhibition of PfSUB1 secretion (Figure 33 and 34), at least in some cells in the case of zaprinast. How then IPP and zaprinast reverse clindamycin induced inhibition of PfSUB1 secretion remains a question to be answered.

**Maintenance of the apicoplast**

Interestingly, clindamycin treated parasites can be rescued by the provision of IPP (Figure 26). However, this rescue is only possible for the first half of the second cycle (Figure 26) and provision of the IPP does not rescue the parasites thereafter (Figure 26). This could mean that either the daughter cells inherit reserves of IPP from the
mother cell, or the apicoplast that they inherit, is functional for the time period after invasion to the time point where rescue with IPP is no longer possible.

But bearing in mind that during the second cycle, clindamycin treated parasites undergo normal transformation from rings to schizonts that in-turn do not egress (Figure 21). On one hand, since therefore these parasites can be able to survive without the need of IPP rescue from the beginning of the second cycle (Figure 26) and on the other hand, since they have an abnormal apicoplast (Figure 25), this indicates that they only need a source of IPP. Therefore eliminating the possibility that they inherit reserves of IPP from the mother cell.

Furthermore, antibiotic treated parasites lose the apicoplast and its genome (Ye and DeRisi 2011) and they can continue to survive without this essential organelle under exogenous IPP supply (Ye and DeRisi 2011). In other studies, interruption of proteins/pathways in the apicoplast results in the loss of this organelle (Mazundar et al., 2006; Tawk et al., 2011; Gisselberg et al., 2013) and delayed death of the parasites (Mazundar et al., 2006; Tawk et al., 2011). To survive these apicoplast-less parasites rely also on the provision of exogenous IPP (Gisselberg et al., 2013). Therefore, the parasites inherit a fully functional apicoplast that later becomes abnormal.

Considering that clindamycin induces inhibition of the apicoplast protein translation (Camps et al., 2002) and consequently inhibition of its biogenesis, both during liver (Friesen et al. 2010) and blood stage ((Figures 22 - 25) and Goodman et al., 2007; Ye and DeRisi 2011)), what factor is required to maintain apicoplast biogenesis? In other words, why does its biogenesis become inhibited? And in-fact, looking at the blood stage why does this occur at later time points during the 2nd cycle post clindamycin exposure and not the earlier time points? Not even during the first cycle?

This suggests that *Plasmodium* inherits a fully functional apicoplast and a certain factor (henceforth called factor X). This factor must be continuously synthesised or supplemented for it to maintain the apicoplast biogenesis. This is important in order for the later to perform its biological role of IPP production during the blood stage (Jomaa et al., 1999; Ye and DeRisi 2011) and fatty acid during the liver stages (Yu et
Therefore, based on the difference in terms of the number of daughter cells between the liver and blood stages of *Plasmodium*, averagely 10,000 vs 20 respectively, the reason due to the different effects of clindamycin on these two stages of the parasite cycle is probably due to quantitative differences.

Bearing this in mind, that Factor X is inherited from the mother cell, it therefore becomes depleted during the liver stage by dilution due to its inheritance from 1 parent cell to averagely 10,000 daughter cells. Therefore it must be replenished otherwise if not and as seen in Friesen *et al.*, inhibition of apicoplast biogenesis occurs within the cycle of drug application (2010). As opposed to the liver stage, during the blood stage, one parent cell give rise to averagely 10-32 daughter cells, and each of these (assuming they all invade and rupture) in turn give rise to again averagely 10-32 daughter cells, therefore factor X is not diluted as much as in the former. Consequently it is passed on to a new generation of daughter cells. But at some point, it becomes depleted by further dilution resulting in delayed death. But what could this mysterious factor X be?

The apicoplast Fe-S cluster biosynthesis pathway has been shown to be important for the maintenance of the apicoplast. This is because, it functions to provide Fe-S cofactors to apicoplast based proteins and pathways (i.e. ferredoxin redox system, Lipoate synthesis, tRNA modifications pathway, and the enzyme IspH of the isoprenoid biosynthesis pathway). As a proof, inhibition of this pathway, leads to the loss of the apicoplast and its genome. This is in contrast to the inhibiton of the isoprenoid biosynthesis pathway by the antibiotic fosmidomycin. However, in both cases, IPP supplementation rescues the parasites (Gisselberg *et al.*, 2013). In addition, studies in *Toxoplasma gondii*, a model apicomplexan parasite, implicate the type II fatty acid (FASII) (Mazundar *et al.*, 2006) as well as a phosphotidylinositol 3 monophosphate (PI3P) mediated endosomal vesicular protein trafficking to the apicoplast (Tawk *et al.*, 2011) to play a role in maintaining the biogenesis of the organelle.

Considering that the apicoplast imports a huge bulk of proteins from the nucleus, approximately 545 (Ralph *et al.*, 2004), and that its genome has only 64 genes,
majority being tRNAs and transcription factors (Wilson et al., 1996). Thus, a great majority of proteins are imported from the nucleus to the apicoplast (Waller et al., 1998). Therefore, could it be that, the inability of clindamycin treated parasites to import and process proteins from the nucleus (Goodman et al. 2007; Ye and DeRisi 2011), indicate a defective apicoplast protein import machinery preceding these events? Hence, could the apicoplast protein importation machinery (Waller et al., 1998) complement the above-mentioned pathways to maintain its biogenesis?

In other words, clindamycin induced inhibition of the apicoplast protein translation (Camps et al., 2002) would result in the absence of functional apicoplast proteins. These include part of the protein import machinery i.e. ClpM (formerly known as ClpC or Hsp93) and post-translation modification protein i.e. SufB (Waller and McFadden 2005). This could results in two consequences: inability of the apicoplast to import proteins and perform post-translation modification of imported as well as the expressed proteins. These in general could reduce the functional protein pool in the apicoplast i.e. house keeping and metabolic proteins. The absences of house keeping proteins would inhibit transcription and translation of the apicoplast genome. Consequently this would lead to the inhibition of apicoplast replication, resulting in daughter cells with no apicoplast. These daughter cells therefore become deprived of the essential metabolic products from the apicoplast e.g. IPP during blood stage.

Studies on other plastids implicate ClpC to play a role in protein import (Constan et al., 2004; Kovacheva et al., 2007) and iron homeostasis (Wu et al., 2010). Plants that have a mutant ClpC exhibit chlorosis, but they can be rescued by expression of wild type clpc1 and or watering with water enriched with iron (Wu et al., 2010). These mutants have defects in the proteins needed to import iron from the cytoplasm into the chloroplast (Wu et al., 2010). Thus defective clpc1 results in the inability to import iron-importing proteins into the chloroplast (Wu et al., 2010). Therefore perhaps, this mysterious factor that is required to maintain the apicoplast could be ClpM.

To summarise, the data from the first part of this study demonstrate that by producing IPP, the apicoplast plays a role during egress. These findings expand the previous understood model on the antibiotics mode of action in P. falciparum to involve the inhibition of egress machinery during erythrocytic schizogony (Figure 45).
Part 2

Acyldepsipeptides induced inhibition of apicoplast biogenesis and *P. falciparum* growth

Based on the bacterial toxicity of ADEPS (Brötz - Oesterhelt *et al.*, 2005; Sass *et al.*, 2011) and the bacterial nature of the apicoplast (Wilson *et al.*, 1996), the aim of the second part of this study was to determine whether ADEPS can inhibit biogenesis of the apicoplast and therefore whether they can act as antimalarial drugs. This was tested by studying the function and biogenesis of the apicoplast in *P. falciparum* under treatment with ADEPS (Figures 14 and 15 above, under materials and methods).

ADEP 1 at 10 µg/ml did not have any effect on the biogenesis and function of the apicoplast (Figure 41) and therefore it did not inhibit parasite growth (Figure 38) but at 30 µg/ml ADEP1 inhibited parasite growth at around 72 hours post invasion (Figure 38). On the contrary, ADEP4 inhibited parasite growth and development at similarly low and high concentrations (Figure 39). However, this inhibition was different such that ADEP4 at 30 µg/ml demonstrated immediate inhibition, whereby, upon invasion, the merozoites were able to transform into rings but got arrested within 24 hours. However, ADEP4 at 10 µg/ml interestingly induced a phenotype characterized by slow parasite growth with a lag phase of 12-24 hours (Figure 39 - 40). As compared to the control, the biogenesis of the apicoplast in these parasites was inhibited (Figure 41 A and B). At the time point where it should have undergone segregation into the daughter cells it appeared branched (Figure 41 A and B), a phenotype reminiscent of filamentation in bacterial cells treated with ADEPS (Brötz - Oesterhelt *et al.*, 2005; Sass *et al.*, 2011). This suggested that ADEP4 induced inhibition of the apicoplast biogenesis and function.

However, supplementation of its biological function during erythrocytic schizogony by continuous exogenous supply of IPP (Figures 42 and 43) or knocking it out chemically did not rescue the parasite from the effects of ADEP4 (Figures 42, 43 and 46). This proved that despite its bacterial nature, *in P. falciparum*, the apicoplast is not the only target of ADEPS, rather, the target are apicoplast dependent and apicoplast independent (Figure 46).
Furthermore, based on its ancestral origin, the apicoplast originated from cyanobacteria which is a gram negative bacteria (Waller and McFadden 2005) and ADEPS have only been shown to act on gram positive bacteria (Brötz - Oesterhelt et al., 2005; Sass et al., 2011). This indicates perhaps ADEPS are unable to penetrate the membranes of the apicoplast. But in this case how are ADEPS acting on *P. falciparum*?

Considering that ADEPS in bacteria target caseinolytic protease (ClpP) (Brötz - Oesterhelt et al., 2005; Sass et al., 2011) and since *P. falciparum* has a ClpP too (Bakkouri et al., 2010), it might be possible that its also targeted by ADEP4. Therefore it might induce cellular damages within the host cell too. This would then result in the observed slowed parasite’s cycle during blood stage (Figures 39, 40, 41 and 42).

Because in other studies; DNA damage is associated with slow growing cells compared to fast or normal growing cells (Van Dijk et al., 2015). Indeed the antibiotic resveratrol by inducing DNA damage in bacteria cells stimulates DNA damage repair processes. This in-turn acts by inhibiting cell division. The cells get arrested as filamented structures (Hwang and Lim 2014). Threfore, it might be also possible that ADEP4 is simply toxic to *P. falciparum*. 
During blood stage of *P. falciparum*, normally, first a merozoite invades an erythrocyte, then it undergoes transformation and multiplication which is coupled to the biogenesis of the apicoplast, that grows, branches and get segregated to the daughter merozoites. These eventually egress and continue the cycle (A). According to the current model on the antibiotic mode of action, exposure to apicoplast's protein translation inhibitory antibiotics post invasion during blood stage, results in normal apicoplast biogenesis and parasite growth during the first cycle. The parasite produces merozoites that egress, invade uninfected erythrocytes but the apicoplast biogenesis and function become inhibited, consequently the parasite becomes arrested and does not progress to a new cycle. However, this left a misery regarding the fate of these parasites and the apicoplast as well, which is a different observation as compared to exposing the liver stage to antibiotics before invasion (B). Therefore to reconcile this discrepancy, this study mimicked the experimental set up in the liver stage study (Friesen et al., 2010), by exposing the parasite to an antibiotic called Clindamycin before invasion (C). During the first cycle, parasite development and apicoplast biogenesis was normal; the parasite produced merozoites that egressed, invaded uninfected erythrocytes and started a 2nd cycle. But two things happen: first, the apicoplast biogenesis becomes inhibited; it remains as a small rounded structure meaning that it was not able to grow, branch and segregate. Consequently the daughter cells do not get a copy of it. Therefore it does not perform its function of IPP production and egress. Eventually, these daughter cells become arrested and do not egress. Early (but not late) supply of exogenous IPP (D) or zaprinast (E) to clindamycin treated parasites was interestingly in-turn able to rescue these parasites. This study, therefore expands the previous understood models on the function of the apicoplast and the antibiotics mode of action in *P. falciparum* during erythrocytic schizogony. Additionally, using ADEPS as a new class of antibiotics, this study shows their potential as antimalarial drugs; they are able to induce inhibition of the parasite in a yet unidentified mechanism. This includes inhibiting targets within and outside the apicoplast (F). Therefore they have potential to be used as antimalarial drugs.

**Figure 47 Summary.** During blood stage of *P. falciparum*, Normally, first a merozoite invades an erythrocyte, then it undergoes transformation and multiplication which is coupled to the biogenesis of the apicoplast, that grows, branches and get segregated to the daughter merozoites. These eventually egress and continue the cycle (A). According to the current model on the antibiotic mode of action, exposure to apicoplast’s protein translation inhibitory antibiotics post invasion during blood stage, results in normal apicoplast biogenesis and parasite growth during the first cycle. The parasite produces merozoites that egress, invade uninfected erythrocytes but the apicoplast biogenesis and function become inhibited, consequently the parasite becomes arrested and does not progress to a new cycle. However, this left a misery regarding the fate of these parasites and the apicoplast as well, which is a different observation as compared to exposing the liver stage to antibiotics before invasion (B). Therefore to reconcile this discrepancy, this study mimicked the experimental set up in the liver stage study (Friesen et al., 2010), by exposing the parasite to an antibiotic called Clindamycin before invasion (C). During the first cycle, parasite development and apicoplast biogenesis was normal; the parasite produced merozoites that egressed, invaded uninfected erythrocytes and started a 2nd cycle. But two things happen: first, the apicoplast biogenesis becomes inhibited; it remains as a small rounded structure meaning that it was not able to grow, branch and segregate. Consequently the daughter cells do not get a copy of it. Therefore it does not perform its function of IPP production and egress. Eventually, these daughter cells become arrested and do not egress. Early (but not late) supply of exogenous IPP (D) or zaprinast (E) to clindamycin treated parasites was interestingly in-turn able to rescue these parasites. This study, therefore expands the previous understood models on the function of the apicoplast and the antibiotics mode of action in *P. falciparum* during erythrocytic schizogony. Additionally, using ADEPS as a new class of antibiotics, this study shows their potential as antimalarial drugs; they are able to induce inhibition of the parasite in a yet unidentified mechanism. This includes inhibiting targets within and outside the apicoplast (F). Therefore they have potential to be used as antimalarial drugs.
CONCLUSIONS AND RECOMMENDATIONS

The data from the first part of this study demonstrate that the timing of antibiotic exposure during erythrocytic schizogony of *P. falciparum* has no influence on their effects on the biogenesis and function of the apicoplast and consequently the growth of *P. falciparum*. However, by inhibiting the biogenesis and function of the apicoplast in *P. falciparum* during erythrocytic schizogony, antibiotics inhibit the egress machinery of the parasite (Figure 47). This demonstrates that the apicoplast plays a role in the egress of the parasite. Hence, by targeting these processes new intervention strategies can be generated to fight the malaria burden in the world.

The data from the second part of this study demonstrate that ADEP4 can inhibit *P. falciparum*, however, despite its bacterial nature the apicoplast is not the only target (Figure 47). ADEPS inhibits targets within and outside the apicoplast. Therefore more work is needed to be done to identify the target of ADEPS in *P. falciparum*. 
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Supplementary Data

Supplementary Figure 1 Agarose gel electrophoresis to confirm lack of DNA contamination. As compared to the positive control, cDNA sample amplified did not have a band of 400 bp which is the size of the expected amplicon for the primers pair 1 (Table 2) indicating lack of DNA contamination.
Isopentenyl pyrophosphate links apicoplast function to egress; two different biological processes in *Plasmodium falciparum*

Key Words: *Plasmodium falciparum*, Clindamycin, Isopentenyl pyrophosphate, Zaprinast

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Summary

To clearly delineate developmental events under antibiotic exposure we decided to decouple potentially confounding effects of different time points of exposure. We therefore synchronized drug exposure and parasite cycle in vitro during erythrocytic schizogony of *Plasmodium falciparum*. Exposure of *P. falciparum* to clindamycin before invasion resulted in the inhibition of the apicoplast biogenesis and consequently parasite arrest. These apicoplast-less parasites demonstrated defects in egress, relying on an unusual early supplementation with Isopentenyl pyrophosphate (IPP) or Zaprinast, an indication of apicoplast function in the egress of *P. falciparum*.

Introduction

Human malaria is a disease caused by infection with *Plasmodium*. Through a bite by an infected mosquito, parasite stages known as sporozoites migrate to the liver and initiate liver stage of the parasite life cycle. The parasite undergoes transformation and multiplication, giving rise to about 10,000 merozoites, which get released into the bloodstream, invade erythrocytes and initiate blood stage of the parasite life cycle. The parasite again undergoes transformation into rings, trophozoites and eventually schizonts that produce about 10-32 merozoites, which invade uninfected erythrocytes to continue the blood stage. The parasite also transforms into gametocytes, which after a blood meal end up in a mosquito to continue its life cycle.

Malaria remains a big health problem. During the year 2015, 214 millions cases and 438,000 deaths of malaria were reported, a big reduction by about 1 billion and 6 millions respectively, during the time period from the year 2001 to 2015 (WHO 2015 A). This achievement is mainly due to the use of malaria control interventions; insecticide treated nets, indoor residual spraying and the use of artemisinin-based combination therapies (ACTs) (WHO 2015 A). In the later intervention strategy, artemisinins, which have a short half-life, are combined with other antimalarial drugs with a long-half life. This is in order to reduce chances of the parasites developing resistance towards them and therefore safeguarding their applicability as antimalarial drugs. Never the less, there have been reports of parasite developing resistance to artemisinins and ACTs (Dondorp et al., 2009; Ashley et al., 2014; WHO 2014), which poses a threat to the current development in the fight against malaria. To safeguard these achievements and continue to win the war against malaria other alternative
drugs need to be used. Antibiotics have recently gained importance in the fight against malaria, as a first line defense against artemisinin or ACTs resistant parasites (Dondorp et al., 2009) and in combination with other drugs they are recommended as back-up treatment measures (WHO 2015 B).

The malaria parasite has an organelle known as the apicoplast, a very essential organelle. This is because it hosts a number of metabolic pathways: the type II fatty acid synthesis, non-mevalonate isoprenoid synthesis, heme biosynthesis, Fe-S cluster biosynthesis, lipoate synthesis, tRNA modifications and ferredoxin redox system (Ralph et al., 2004). By supplying the products of these pathways, it plays different stage specific-roles during the life cycle of the parasite. In Plasmodium falciparum, during the mosquito stage it functions to produce heme (Niagara et al., 2013; Ke et al., 2014), liver stage to produce fatty acids (Yu et al., 2008; Vaughan et al., 2009) the blood stage to produce isoprenoid precursors known as isopentenyl pyrophosphate (IPP) (Jomaa et al., 1999; Ye and DeRisi 2011; Wiley et al., 2015).

Owing to its prokaryotic nature (Wilson et al., 1996), it is amenable to inhibition by antibiotics (Goodman et al., 2007). Exposure of P. falciparum to clindamycin post invasion during erythrocytic schizogony results in normal parasite growth and apicoplast development, the parasites are then able to egress, invade erythrocytes but at some point inhibition of the apicoplast biogenesis occurs and consequently parasite growth (Burkhardt et al., 2007; Dahl et al., 2007; Goodman et al., 2007). Likewise, clindamycin exposure during liver stage, starting with the liver invasive stages (sporozoites), results in the parasites developing into full maturity with no difference to controls (Friesen et al., 2010). In stark contrast to the blood stage phenotype, biogenesis of the apicoplast was immediately inhibited and progeny were unable to cause blood stage infection (Friesen et al., 2010). To mimic this experimental setup we determined the effect of clindamycin immediate exposure starting with the parasite’s invasive stage (merozoites) during erythrocytic schizogony. We demonstrate that a functional apicoplast is required for the egress of merozoites during erythrocytic schizogony.

**Results**

**Clindamycin inhibits egress of Plasmodium falciparum blood stages**

We studied the effects of synchronizing clindamycin drug pressure and parasite cycle during erythrocytic schizogony of P. falciparum. We did this by culturing P. falciparum
in vitro and then using cell cultures enriched for schizonts; we isolated merozoites, which we exposed to clindamycin before invasion (Figure 1A). Thus, cell cycle and start of exposure were synchronized (Figure 1A). We then monitored the parasite growth for 2 cycles. At the end of the first cycle clindamycin-exposed parasites were able to egress and invade uninfected erythrocytes. In this second replication cycle, we observed normal differentiation into rings, trophozoites and then into schizonts via bright field microscopy (Figure 1B). However, treated cultures remained arrested at the schizonts stage at a time point when untreated parasites had progressed to the third cycle (Figure 1B). This indicated that clindamycin inhibits egress of the parasite after two cycles.

**Inhibition of egress correlates with inhibition of the apicoplast biogenesis**

Since delayed exposure to clindamycin >6 hours post invasion during erythrocytic schizogony acts by inhibiting apicoplast biogenesis (Camps et al., 2002; Goodman et al., 2007; Ye and DeRisi 2011), we tested what effects; the synchronization of drug pressure and parasite cycle would have on the apicoplast biogenesis (Figure 1A). We assayed the biogenesis of the apicoplast by immunofluorescence assay and fluorescence microscopy. During the first cycle, in both the treated and untreated cultures, the organelle branched and segregated into daughter cells (Figure 2A and B). In contrast to untreated cultures (Figure 2C), towards the end of the second cycle apicoplast biogenesis appeared inhibited in the clindamycin-arrested schizonts (Figure 2D). This indicated that the inhibition of the apicoplast biogenesis and function preceded the inhibition of egress.

**Chemical rescue with isopentenyl pyrophosphate (IPP) complements the apicoplast function and reverses the inhibition of egress**

Antibiotic treated parasites can be rescued by provision of exogenous IPP, the key product of apicoplast biosynthesis in blood stage parasites (Ye and DeRisi 2011). Here, we tested the hypothesis that the pronounced egress defect is due to IPP starvation and thus, can be directly rescued by IPP supplementation. Surprisingly, the egress defect could not be rescued by IPP directly (-2 hours) and partial as well as full rescue required IPP provision at ≥ -8 hours and ≥ -38 hours prior to egress, respectively (Figure 3). Firstly, this suggests that the structural remnant of the apicoplast (Figure 2D and Supplementary Figure 1) is either incompletely or fully
non-functional during the second cycle. More importantly, it raises the curious question of what cellular function(s) IPP governs early on in the second cycle, which if absent, permits morphologically normal maturation yet interferes with parasite egress.

**Inhibition of cyclic nucleotide phosphodiesterase activity by zaprinast forces egress of clindamycin-arrested *P. falciparum* in an unusual manner**

Failure to directly rescue the egress defect by IPP led us to attempt to induce productive egress, i.e. egress of invasion-competent merozoites, by an alternative chemical option or simply, mechanically. First, we used zaprinast, a previously described inhibitor of cyclic nucleotide phosphodiesterase activity (Collins *et al.*, 2013). Zaprinast-induced surge of intracellular cGMP (Collins *et al.*, 2013) would accelerate egress of invasion-competent merozoites when added to mature schizonts. We confirmed that zaprinast, when added at 43 hours to untreated schizonts, accelerated egress (data not shown) and did not block the progression to the next cell cycle (Figure 4A). Next, we attempted to force egress of clindamycin-arrested schizonts with zaprinast, postulating that this may allow cell cycle progression. However, we observed a pattern reminiscent of rescue with IPP (Figure 3), such that only induction started during the first half of the 2nd cycle resulted in a significant proportion of parasites progressing into the 3rd cycle (Figure 4B). Strikingly, no premature egress was seen in clindamycin treated cultures upon exposure to zaprinast at Hour 84 (data not shown).

**Merozoites mechanically released from clindamycin-arrested schizonts are non-invasive**

To mechanically force egress of the clindamycin-arrested schizonts we ruptured the arrested schizonts using a syringe filter (Boyle *et al.*, 2010). First, we observed morphological normal merozoites (data not shown). However, these merozoites were unable to invade (Figure 5). Taken together, these data demonstrate that clindamycin-induced inhibition of apicoplast biogenesis and function results in defective egress and invasive capacities of 2\(^{nd}\) generation (F2) progeny.
**Clindamycin-induced inhibition of apicoplast function interferes with the secretion of Pfsub1, an essential step in the egress pathway**

From the above findings, we concluded that either an essential biochemical signaling pathway and/or components of the egress machinery are dysfunctional in these parasites. Because of the chemical refactoriness of egress-deficient parasites (which can mechanically be released), we asked ourselves whether this correlates with alterations in a known essential component of the egress machinery. Both the natural egress signal and zaprinast induce egress by activating protein kinase G (PKG) (Collins *et al*., 2013). PKG in-turn functions to regulate the secretion of *Plasmodium falciparum* subtilisin like serine protease-1 (PfSUB1) (Collins *et al*., 2013) from exonemes (Yeoh *et al*., 2007). Because of the central and essential role of PfSUB1 in parasite egress and invasion (Koussis *et al*., 2009; Collins *et al*., 2013), we determined its sub-cellular localization. Untreated cultures displayed a diffuse signal, characteristic of secreted PfSUB1 (Figure 6A and supplementary figure 2A). However, treated cultures had punctate signal, indicating lack of secretion of PfSUB1 (Figure 6B and supplementary Figure 2B). This was reversed on early treatment with IPP (Figure 6C and supplementary figure 2C) or zaprinast (Figure 5D and supplementary Figure 2D). Upon zaprinast treatment a normal, diffuse anti-PfSUB1 staining was observed in a subset of cells, correlating roughly with the proportion of parasites rescued by zaprinast. We thus conclude that clindamycin-induced inhibition of the apicoplast biogenesis and function interferes with the secretion of PfSUB1, suggesting dysfunctional egress signaling in these parasites.

**Discussion**

By exploiting the prokaryotic nature of the apicoplast (Wilson *et al*., 1996), antibiotics can inhibit its house keeping functions or the metabolic pathways it hosts (Waller and McFadden 2005; Goodman *et al*., 2007). They however, produce different effects during the life cycle of the malaria parasite. During the blood stage, exposure to antibiotics post invasion leads to a delayed effect on the biogenesis of the apicoplast and inhibition of the parasite (Burkhardt *et al*., 2007; Dahl *et al*., 2007; Goodman *et al*., 2007). Similarly, antibiotics inhibit the apicoplast biogenesis and parasite growth during the liver stage, but on the contrary, they produce an immediate effect (Friesen *et al*., 2010). Therefore, to clarify this discrepancy, we mimicked the experimental design used during the liver stage (Friesen *et al*., 2010), and we set up a clear-cut
experiment during the blood stage by starting the exposure of merozoite to clindamycin before invasion. We show that during blood stage, a functional apicoplast is required during egress (Figure 1 and 2).

According to the current model on the antibiotic mode of action during blood stage of *P. falciparum*; exposure to apicoplast’s protein translation inhibitory antibiotics post invasion, results in normal apicoplast biogenesis and parasite growth during the first cycle, the parasite produces merozoites that egress, invade uninfected erythrocytes but the apicoplast biogenesis and function becomes inhibited, consequently the parasite becomes arrested and does not progress to a new cycle (Surolia *et al.*, 2004; Burkhardt *et al.*, 2007; Dahl *et al.*, 2007; Goodman *et al.*, 2007; Ye and DeRisi 2011). This left a misery regarding the fate of these parasites. Supply of exogenous IPP to antibiotic treated parasites was interestingly in-turn able to rescue these parasites (Ye and DeRisi 2011). Therefore IPP was sufficient to uncouple apicoplast from parasite development demonstrating that during the blood stage, the apicoplast mainly functions to produce IPP (Jomaa *et al.*, 1999; Ye and DeRisi 2011). IPP inturn functions in the isoprenylation of Rab proteins, phosphoinositide metabolism and vesicular trafficking for the biogenesis of the food vacuole (Howe *et al.*, 2013). Using an approach based on synchronising-drug-exposure-and-parasite-cycle (Figure 1A), we show that during antibiotic-induced delayed death the parasites develop to late schizonts with second-generation merozoites (Figure 1B) but due to defects in the egress machinery (Figure 6) these merozoites are not able to egress (Figure 1B). We therefore expand the previous understood models on the function of the apicoplast and the antibiotics mode of action in *P. falciparum* during erythrocytic schizogony (Figure 7).

Egress is a tightly regulated process, according to the current model, it requires cGMP-dependent Protein Kinase G (PKG) (Collins *et al.*, 2013), which functions by regulating phosphoinositide metabolism and intracellular calcium ion levels (Brochet *et al.*, 2014). An increase in intracellular Ca$^{2+}$ in the cytoplasim is required during egress (Agarwal *et al.*, 2013; Collins *et al.*, 2013; Glushakova *et al.*, 2013) to activate the exocytosis of *P. falciparum* subtilisin-like protein 1 (PfSUB1) (Agarwal *et al.*, 2013; Collins *et al.*, 2013) from exonemes to the parasitophorous vacuole (Yeoh *et al.*, 2007). PfSUB1 in-turn proteolytically mediates the activation of other proteins required for egress (serine-type SERA proteases (Collins *et al.*, 2013) and *P.*
*P. falciparum* perforin-like proteins (PfPLPs) (Garg *et al.*, 2013)) and invasion (merozoite surface proteins 1, 6 and 7 (MSP1, 2 and 7) (Koussis *et al*. 2009)). Inhibition of the apicoplast biogenesis and function by clindamycin inhibits PfSUB1 secretion and consequently egress of the parasite (Figure 1,2 and 6). IPP was able to rescue the parasites from the egress arrest (Figure 3) by reversing this effect (Figure 6C and supplementary figure 2C). Based on the fact that IPP functions in the isoprenylation of Rab proteins, phosphoinositide metabolism and vesicular trafficking for the biogenesis of the food vacuole (Howe *et al.*, 2013). Therefore, due to the interdependence between Rab proteins and phosphoinositide metabolism (Alberts *et al.*, 2008; Jean and Kieger 2012), clindamycin induced shortage of IPP inhibits isoprenylation of Rab proteins and phosphoinositide metabolism (Howe *et al.*, 2013). The latter can therefore not respond to PKG regulation (Brochet *et al.*, 2014). Early supply of IPP can circumvent these effects and therefore rescue the parasite (Figure 3 and 6C).

Zaprinast however, was able to rescue some parasites from the egress arrest (Figure 4B) by inducing secretion of PfSUB1 in some of the parasites (Figure 6D and supplementary figure 2D). This can be explained by considering the fact that egress of *P. falciparum* can be induced chemically by zaprinast (Collins *et al.*, 2013) which acts to activate PKG by inhibiting cGMP phosphodiesterase (Collins *et al.*, 2013), eventually activating Ca$^{2+}$ signaling (Taniguchi *et al.*, 2006; Brochet *et al.*, 2014) and consequently leading to secretion of PfSUB1 (Collins *et al.*, 2013).

The different observations regarding the delayed effects of antibiotics during the blood stage (Surolia *et al.*, 2004; Burkhardt *et al.*, 2007; Dahl *et al.*, 2007; Goodman *et al.*, 2007; Ye and DeRisi 2011) as opposed to immediate effects during the liver stages of the parasites (Friesen *et al.*, 2010), can be attributed to result from quantitative reasons due to the requirement of a high metabolism to form/maintain about 10,000 daughter cells during the liver stage as opposed to the blood stages which forms just about 10 daughter cells. Therefore, inhibition of the apicoplast biogenesis cannot be tolerated during the liver stage, however, during the blood stage, the parasite tolerates inhibition of protein translation in the apicoplast during the first cycle, but during the second cycle this cannot be tolerated. This indicates that there is a factor that is required to maintain biogenesis of the apicoplast but it becomes highly diluted and therefore depleted as a result of massive branching of the apicoplast required for its segregation to the 10,000 merozoites during the liver
stage. However, during the blood stage the fewer merozoites formed do not require such a high dilution of this factor. Therefore, the apicoplast biogenesis is not affected during the first cycle. But during the second cycle this factor becomes further diluted and depleted. Consequently, the apicoplast biogenesis becomes inhibited and therefore it cannot be segregated to the second-generation merozoites (Figure 2). These become deprived of the apicoplast and the essential functions that it fulfills (Figure 2,3,6 and 7).

The limitations of our study are that: we did not determine the state of development of the clindamycin-arrested schizonts, whether the merozoites are fully developed, whether they have parasite plasma membranes around them, as well as to investigate in detail the state of PfSUB1 in clindamycin-arrested schizont.

This study clarifies the mode of action of antibiotics, which are currently components of rescue treatments for artemisinin resistant infections (Dondorp et al., 2009). The practical implication of these findings is that antibiotics could be used to dissect the egress pathway.

**Experimental Procedures**

**Cell Culture**

*Plasmodium falciparum* lab strain 3D7 was cultured *in vitro* according to Trager and Jensen (1976) with a few modifications. Briefly, *P. falciparum* was cultured in Human O+ erythrocytes at 5% haematocrit in culture medium containing RPMI 1640 (Invitrogen) supplemented with (L glutamine and HEPES), 10% Albumax II (Invitrogen), 2% Human AB+ serum, 1% Hypoxanthine (ccpro) and 0.05% Gentamicin (Invitrogen) in 92% N₂, 5% CO₂, 3% O₂. Cultures development and growth were then followed by examination and counting parasitemia, using giemsa stained thin smears.

**In vitro Drug Exposure Before Invasion**

Merozoite isolation was done according to Boyle *et al.*, (2010) with modifications. Briefly, *P. falciparum* cultures were tightly synchronized with D-Sorbitol (Lambros and Vanderberg 1979) and heparin (Boyle *et al.*, 2010). Then at 40 - 42 hours post invasion the cultures were incubated with E64 (trans - Epoxysuccinyl - L - leucylamido (4 - guanidino) butane (Sigma Aldrich) for 5-8 hours. The cultures were then filtered in incomplete culture medium through 1.2 µm Syringe filters (Sartorius).
Equal portions of the merozoites filtrate were used to initiate different cultures that were treated as following: One portion of the filtrate was treated with 50nM clindamycin before invasion. Another portion of the filtrate was used to set up a negative control in which the merozoites were not exposed to drug treatment. Growth and development of the cultures was then followed by examination using giemsa stained thin smears and light microscopy for two cycles.

**Rescue with Isopentenyl Pyrophosphate (IPP)**

Rescue of clindamycin-arrested parasites with IPP was done according to Yeh and DeRisi (2011) with modifications. Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then they were treated by adding 200 µM of IPP (Sigma Aldrich) at different times post invasion, namely at 48, 60, 72, 84 and 96 hours. As controls, one group of cultures exposed to clindamycin before invasion and the negative control were not treated with IPP.

**Induction of Egress with Zaprinast**

Induction of egress in clindamycin-arrested parasites with Zaprinast was done according to Collins et al., (2013) with modifications. Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then they were treated by adding 100 µM of Zaprinast (Sigma Aldrich) at different times post invasion, namely: at 48, 60, 72, 84 and 95 hours. As controls, one group of cultures exposed to clindamycin before invasion and the negative control were not treated with Zaprinast.

**Immunofluorescence Assay**

Different groups of cultures were exposed to clindamycin before invasion as described above. Then immunofluorescence assay (IFA) was done as described by Tonkin et al., (2004) and Collins et al., (2013) with few modifications. Briefly, cells were harvested from cultures and washed with 1X PBS. They were then fixed. For apicoplast IFA, this was done using 4% Paraformaldehyde (AppliChem) and 0.75% Gluteraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at 37°C. For PfSUB1 IFA, this was done using 4% Paraformaldehyde (Agar Scientific) and 0.0075% Gluteraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at room temperature. They were subsequently washed with 1x PBS. Thin smears were then made on Poly - L -
Slides (Sigma Aldrich). Permeabilization was then done. For apicoplast IFA, this was done using 0.2% Triton X-100 (Carl Roth) in 1X PBS for 30 minutes at room temperature. For PfSUB1 IFA, this was done using 0.1% Triton X-100 (Carl Roth) in 1X PBS for 10 minutes at room temperature. The cells were subsequently washed with 1X PBS. Blocking was then done. For apicoplast IFA, this was done using Blocking Aid Solution (Invitrogen) for 1 hour at room temperature in a humidified chamber. For PfSUB1 IFA, this was done using Bovine Serum Albumin (Roth) for 1 hour at room temperature in a humidified chamber. Primary antibody was then applied: For apicoplast IFA, Anti-ACP primary antibody diluted in Blocking Aid Solution (Invitrogen) (1:250) was used and incubated for 2 hours in a dark humidified chamber at room temperature. For PfSUB1 IFA, Anti-PfSUB1 primary antibody diluted in PBS (Sigma Aldrich) /0.5% (v/v) Tween 20 (Sigma Aldrich) /1% (w/v) BSA (Roth) (1:250) was used and incubated for 1 hour in a humidified dark chamber at room temperature. The cells were then washed three times in 1X PBS. Secondary antibody was then applied and incubated for 1 hour in a dark humidified chamber at room temperature. For apicoplast IFA, Alexa Fluor 546 (Invitrogen) diluted in Blocking Aid Solution (Invitrogen) (1:1000) was used. For PfSUB1 IFA, Alexa Flour 548 (Invitrogen) diluted in PBS (Sigma Aldrich) /0.5% (v/v) Tween 20 (Sigma Aldrich) /1% (w/v) BSA (Roth) (1:1000) was used. The cells were incubated for 1 hour in a humidified dark chamber at room temperature. They were then washed three times with 1X PBS. Hoechst 33342 (Invitrogen) diluted in 1x PBS (1:1000) was then added and incubated for 5 minutes in a humidified dark chamber at room temperature. The cells were then washed three times in 1X PBS. They were then mounted with Pro- long Gold mounting solution (Invitrogen). The samples were then examined with a fluorescence microscope (Zeiss Observer.Z1).

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Conflict of Interest
We wish to state that there is no conflict of interest.

Author Contributions
SB contributed to the design of the study, the interpretation of the data and writing of the manuscript. DNN contributed to the data acquisition, data analysis and writing of the manuscript.

References


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Figure Legends

**Figure 1 Response of P. falciparum to Clindamycin.** (A). *P. falciparum* blood stages were cultured *in vitro*. The cultures were then tightly synchronized and used to isolate merozoites. These merozoites were subsequently treated with clindamycin
before invasion. The cultures were then monitored at 12-hour intervals for 2 cycles by light microscopy. A control was set up with no drug exposure. (B). Thin smears of *P. falciparum in vitro* cultures during the second cycle post invasion. The bottom row represents culture treated with clindamycin and the top row is the control. Note the persistence of arrested schizonts beyond the end of the second cycle (hour 84) in clindamycin treated parasites into the third cycle (hour 96). Note that the average duration of the erythrocytic cell cycle in our culture of the 3D7 strain was 43 hours.

**Figure 2 Effect of clindamycin on *P. falciparum* apicoplast biogenesis.** Immunofluorescence assay pictures of the apicoplast in *P. falciparum* blood stages. Apicoplast structures were visualized by an anti-acyl carrier protein (ACP), an essential ‘signature’ protein of the apicoplast. During, the first cycle in both the treated and the un-treated cultures biogenesis of the apicoplast was not affected (A and B). During the second cycle, biogenesis of the apicoplast was arrested in the clindamycin exposed, but not in control (C), cultures (D).

**Figure 3 IPP rescue of clindamycin treated parasites at different time points post invasion.** Importantly, only parasites treated with IPP during the first half of the second cycle were able to progress to a third cycle (TR H48, TR H60, TR H72 and TR H78) but at different proportions as compared to the control. **Key:** TR48, TR60, TR72, TR78, TR84, TR96: Test sample treated with IPP at 48, 60, 72, 78, 84 and 96 hours post invasion respectively. The experimental replicates are a minimum of 3 independent experiments. Error bars indicate standard deviation.

**Figure 4. Zaprinast rescue of clindamycin arrested schizonts at different time points post invasion.** (4A) Zaprinast induced egress of untreated late schizonts did not interfere with the invasiveness of the merozoites (4B) indicating what we would expect to see at the end of the cycle under zaprinast treatment. Treatment of clindamycin-arrested schizonts with Zaprinast at 48 hours post invasion was able to rescue the parasites. **Key:** ZH48, ZH60, ZH72, ZH84 and ZH95: Test samples treated with clindamycin before invasion and then with Zaprinast at 48, 60, 72, 84 or 95 hours post invasion. Test: samples treated with clindamycin only. The experimental replicates are a minimum of 3 independent experiments. The statistical test used is Kruskal-Wallis rank test. Error bars indicate standard deviation.
Figure 5 Invasion assay, determined by counting ring stages at 12 hours post invasion. As compared to the control merozoites released by mechanical rupture of clindamycin-arrested schizonts were not able to invade erythrocytes. The experimental replicates are four independent experiments. The statistical test used is Kruskal-Wallis rank test. Error bars indicate standard deviation.

Figure 6 Representative images from immunofluorescence assays of PfSUB1, a known component of the egress machinery, at the end of the second cycle. (A) In control cultures, the signal was diffuse, indicating regular secretion of PfSUB1. (B) In contrast, in clindamycin treated samples the signal was punctate indicating lack of PfSUB1 secretion. (C) This was reversed on rescue with IPP or (D) zaprinast (both drugs applied at hour 48, i.e., 37 hours prior to egress). About half of the cells exposed to zaprinast early during the second cycle displayed normal PfSUB1 staining pattern, indicating a correlation with the subset of parasites rescued by early exposure to zaprinast (Fig. 4B).

Figure 7 A model for the role of the apicoplast during the egress of *P. falciparum*. According to the previous understood model, antibiotics inhibit biogenesis of the apicoplast and therefore its function in IPP production (B). This study further shows that antibiotics act during erythrocytic schizogony by inhibiting the egress of the parasite (C). This effect can be reversed by early rescue with IPP (D) or zaprinast (E). In demonstrating that, by producing IPP a functional apicoplast is required for PfSUB1 secretion and eventually egress during erythrocytic schizogony this study therefore expands the current understanding of its biology during erythrocytic schizogony.

Supplementary Figure 1 Effect of clindamycin on *P. falciparum* apicoplast biogenesis. Immunofluorescence assay pictures of the apicoplast in *P. falciparum* blood stages at hour 66 during the second cycle. Apicoplast structures were visualized by an anti-acyl carrier protein (ACP), an essential 'signature' protein of the apicoplast. In both the treated (A) and the clindamycin-treated cultures (B) biogenesis of the apicoplast was not affected.
Supplementary Figure 2A Immunofluorescence assay of PfSUB1 at the end of the second cycle. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* untreated with clindamycin before or after invasion (C). In the various cells observed the appearance of PfSUB1 ranged from absent, weak to diffuse.

Supplementary Figure 2B Immunofluorescence assay of PfSUB1 at the end of the second cycle. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* treated with clindamycin before invasion (T). In the various cells observed the appearance of PfSUB1 was strong and punctate.

Supplementary Figure 2C Immunofluorescence assay of PfSUB1 at the end of the second cycle. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* treated with clindamycin before invasion and then with zaprinast at 48 hours post invasion (Z). The appearance of PfSUB1 was strong and punctate in some cells and in others it ranged from absent, weak to diffuse.

Supplementary Figure 2D Immunofluorescence assay of PfSUB1 at the end of the second cycle. Shown here are immunofluorescence assay pictures of the localisation of PfSUB1 in *P. falciparum* treated with clindamycin before invasion and then with IPP at 48 hours post invasion (I). The appearance of PfSUB1 ranged from weak to diffuse.
A

P. falciparum culture and synchronisation

Merozoite filtration

Clindamycin

Merozoite filtrate

Erythrocytes

Cell culture

B

2nd cycle

Hour 43

Hour 60

Hour 72

Hour 84

Hour 86

3rd cycle

Hour 96

Untreated

Clindamycin
Current model of antibiotics mode of action during erythrocytic schizogony

1st cycle

A + Antibiotics

B + Antibiotics

? Fate of the apicoplast during the 2nd cycle

2nd cycle

Invasion

New model on antibiotics mode of action during erythrocytic schizogony

1st cycle

C + Antibiotics

D + Antibiotics

E + Antibiotics

2nd cycle

IPP Zaprinast

IPP Zaprinast

IPP Zaprinast

3rd cycle

Egress defect

Invasion

No Invasion

Invasion (30% of A)

Egress defect
Acyldepsipeptides slows down the cycle of *Plasmodium falciparum* during erythrocytic schizogony

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ABSTRACT

Background

We tested an experimental class of antibiotics called acyldepsipeptides (ADEPS) on whether they can target the biogenesis or function of the apicoplast in *P. falciparum* during erythrocytic schizogony and therefore whether they have antimalarial activity.

Results

Despite ADEPS not being able to target the apicoplast biogenesis or function they were able to inhibit growth of *P. falciparum* interestingly inducing a phenotype characterized by slow parasite growth.

Conclusion

ADEPS have antimalarial activity

Key Words

Malaria, *Plasmodium falciparum*, Apicoplast, Antibiotics, ADEPS
Background

Malaria being a global health disaster necessitates development of intervention strategies. Parasites development of resistance to antimalarial drugs is however a huge hindrance to the fight against malaria. Therefore there is need for the development of new antimalarial drugs not only for combination therapy but also as a back up.

The malaria parasite harbors the apicoplast, an organelle of prokaryotic origin [1]. This organelle has essential functions and is an established drug target [2, 3, 4, 5, 6, 7, 8, 9,10]. Indeed a number of classical antibiotics are able to inhibit its biogenes or function and consequently inhibiting the parasite [3, 11, 12, 13, 14, 15, 7].

Acyldepsipeptides (ADEPS) are 6 different compounds (ADEPS 1 to 6) produced from a parent compound called A54556 complex [16, 17]. In bacteria, ADEPS act via a unique mode of action [17]. They bind to ClpP protease, activating it uncontrollably [17, 18]. The unregulated ClpP then preferentially targets bacterial cell division machinery [17, 19].This results in the inhibition of bacterial cell division, with parasites ending up as filamented or swollen cells [19, 17]. Since the apicoplast is bacterial in nature, we therefore determined whether ADEPS can target the apicoplast and inhibit the growth of *P. falciparum*.

RESULTS

**ADEP 4 slows down the cell cycle of Plasmodium falciparum**

To determine whether ADEPS can inhibit *P. falciparum*, we exposed the parasites *in vitro* to different types of ADEPS: ADEP 1 and 4. ADEP 1 did not show any activity against *P. falciparum* (data not shown). But ADEP 4 demonstrated different effects, at 30 µg/ml it inhibited parasite growth within 24 hours during the first cycle and at 10 µg/ml it induced slow parasite growth as depicted by Low parasitemia (Figure 1A)
and delayed parasite cycle during blood stage (Figure 1B). This indicated that ADEP1 had no effect against *P. falciparum* but ADEP4 was active.

**ADEP4 induces filamentation of the apicoplast**

The apicoplast is bacterial in nature [1] and since it cannot be formed de novo it has to be inherited from the mother cell to the daughter cell. Therefore it undergoes biogenesis whereby it starts as a tiny structure which grows, branches and eventually gets segregated to the daughter cells, which later egress and invade uninfected erythrocytes [2]. ADEP4 demonstrates effects against *P. falciparum* (Figure 1), therefore to determine whether the apicoplast was the target; we studied its biogenesis using immunofluorescence assay at the end of the first and second cycle, under treatment with ADEP 1 or 4 at 10 µg/ml. The apicoplast biogenesis under ADEP 1 was not affected both during the end of the first and second cycle (Fig. 2A and 2B). However, under ADEP 4 treatments, at the time point whereby it should have undergone segregation i.e. at the end of the first and second cycle, it remained still branched (Fig. 2A and 2B). This morphology is typical of younger parasite stages (i.e. trophozoites). This confirms the delayed parasite cycle during the blood stage and indicates inhibition of the apicoplast biogenesis.

**Chemical complementation with IPP does not rescue ADEP4 treated parasites**

The apicoplast functions during the erythrocytic schizogony to produce isopentenyl pyrophosphate (IPP) [3, 7]. Therefore, to determine whether this effect of ADEP4 on *P. falciparum* (Figure 1) is through the inhibition of the apicoplast function; we supplemented the apicoplast function by treating *P. falciparum* with ADEP4 under continuous exogenous IPP provision. Provision of IPP did not rescue the parasites from ADEP4 treatment at 10 ug/ml and 30 µg/ml (Figure 3A). These parasites appeared to lag even much behind (Figure 3B) as compared to when treated with ADEP4 only (Figure 1B). This indicates that, ADEP4 does not affect the function of
the apicoplast in IPP production but ADEPS may have other unknown targets.

Chemical knockout of the apicoplast does not rescue ADEP4 treated parasites

Cellular materials and organelles that cannot be synthesised denovo have to be replicated and passed on to the daughter cells. And because ADEP4 is affecting the apicoplast biogenesis and its segregation, therefore to determine whether this was the reason behind the effects of ADEPS (Figure 1 and 2), we chemically knocked out the apicoplast and maintained these parasites under continuous exogenous IPP provision (Figure 4A and B). We then treated the parasites with ADEP4 at 10 and 30 ug/ml under continuos IPP provision. However, parasite growth was still inhibited (Figure 4C). This confirms that the target of ADEP4 is not the apicoplast.

DISCUSSION

Based on the antibacterial activity of ADEPS [17, 19] and the bacterial nature of the apicoplast [1], we determined whether ADEPS can inhibit biogenesis of the apicoplast and therefore whether they can act against *P. falciparum*. ADEP1 had no activity against *P. falciparum* (data not shown). However, ADEP4 demonstrated immediate parasite inhibition at high concentration and at low concentration, it induced slow parasite growth (Figure 1A and B). Since this effect however was not due to it targeting the function of the apicoplast in IPP production (Figures 3 and 4), a possible hypothesis on how ADEP4 exert its effect on *P. falciparum* would be that: ADEPS are unable to penetrate the membranes of the apicoplast. This is because, based on its ancestral origin, the apicoplast originated from a cyanobacteria which is a gram negative bacteria [2] and ADEPS have only been shown to act on gram positive bacteria [17, 19]. Considering that ADEPS in bacteria target caseinolytic protease (ClpP) [17, 19] and since *P. falciparum* has a ClpP too [20], it might be possible that its also targeted by ADEP4. Therefore it might induce cellular damages within the host cell as well and this would then result in the observed effects (Figure
Conclusion

We demonstrate that ADEPS can inhibit growth of *P. falciparum* and therefore they have potential to be used as antimalarial drugs.

Materials and Methods

Cell Culture

*Plasmodium falciparum* lab strain 3D7 was cultured *in vitro* according to the protocol by Trager and Jensen [21]. Briefly, *P. falciparum* was cultured in Human O+ erythrocytes at 5% haematocrit in culture medium containing RPMI 1640 (Invitrogen) supplemented with L glutamine and HEPES, 10% Albumax II (Invitrogen), 2% Human AB+ serum, 1% Hypoxanthine (ccpro) and 0.05% Gentamicin (Invitrogen) in 92% N₂, 5% CO₂, 3% O₂. Parasite growth was then followed by examination and counting parasitemia using giemsa stained thin smears.

In vitro Drug Exposure Before Invasion

Merozoites were isolated according to the protocol by Boyle *et al.*, [22]. Briefly, *P. falciparum* cultures were tightly synchronized with D-Sorbitol [23] and heparin [22]. After 40 - 42 hours post invasion the cultures were treated with E64 (Sigma Aldrich) and incubated for 5-8 hours. The cultures were then filtered in incomplete culture medium through 1.2 µm syringe filter (Sartorius). Equal portions of the merozoites filtrate were used to initiate different cultures that were treated before invasion with either: ADEP 1 at 10 µg/ml and 30µg/ml, ADEP 4 at 10 µg/ml and 30µg/ml or 50nM clindamycin. A portion of the filtrate was used to set up a negative control in which the merozoites were not exposed to any drug treatment. Growth and development of the cultures was then followed by examination using giemsa stained thin smears and light microscopy for two cycles.
Rescue with Isopentenyl Pyrophosphate

Cultures of *P. falciparum* were treated with IPP according to the protocol by Yeh and DeRisi [7]. Different groups of cultures were first exposed to ADEPS before invasion as described above. They were then treated by adding 200 µM of IPP (Sigma Aldrich) at 48 hours post invasion. As controls, one group of cultures exposed to ADEPS before invasion and the negative control were not treated with IPP.

Chemical Knock - Out of the apicoplast

Chemical knock-out of the apicoplast was performed according to the protocol by Yeh and DeRisi [7]. Briefly, merozoites were isolated as described above. They were subsequently treated with 50nM clindamycin before invasion and used to initiate cultures. Starting at 48 hours post invasion the cultures were treated by adding 200 µM of IPP (Sigma Aldrich). Then after 48 hours, the culture media was replaced with new media and IPP. As controls, one group of culture exposed to clindamycin before invasion and the negative control were not treated with IPP.

Localization assays of Apicoplast

Localization assays of the apicoplast and PfSUB1 were performed using immunofluorescence assay (IFA) as described by Tonkin et al., [23]. Briefly, different groups of cultures were exposed to clindamycin before invasion as described above. Then cells were harvested from these cultures accordingly (Apicoplast after every 12 hours for 2 cycles and PfSUB1 towards the end of the second cycle). They were then washed with 1X PBS and fixed using 4% Paraformaldehyde (AppliChem) and 0.75% Gluteraldehyde (Sigma Aldrich) in 1X PBS for 30 minutes at 37°C. After fixation, the cells were washed with 1x PBS and thin smears were made on Poly - L - Slides (Sigma Aldrich) and permeabilized by treating the cells with 0.2% Triton X-100 (Carl Roth) in 1X PBS for 30 minutes at room temperature. The cells were washed with 1X
PBS and blocked by treating the cells with Blocking Aid Solution (BAS) (Invitrogen) for 1 hour at room temperature in a humidified chamber. Anti-ACP primary antibody diluted 1:250 in Blocking Aid Solution (Invitrogen) was used. The cells were then incubated for 1-2 hours in a dark humidified chamber at room temperature. They were then washed three times with 1X PBS. Alexa Fluor 546 (Invitrogen) diluted 1:1000 in Blocking Aid Solution (Invitrogen) was then applied as a secondary antibody. The cells were then incubated for 1 hour in a dark humidified chamber at room temperature and then washed three times with 1X PBS. Hoechst 33342 (Invitrogen) diluted 1:1000 in 1x PBS was then added and the cells were incubated for 5 minutes in a humidified dark chamber at room temperature. The cells were then washed three times with 1X PBS. Finally, the cells were mounted with Pro-long Gold mounting solution (Invitrogen) and examined with a fluorescence microscope (Zeiss Observer.Z1).

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**Figure legends**

**Figure 1 Growth inhibition assay of ADEP4 on P. falciparum.** A). ADEP4 at 10 ug/ml (ADEP 4 10) did not inhibit parasite growth while ADEP4 at 30 ug/ml (ADEP4 30) inhibited parasite growth within the first cycle. Error bars represent standard deviation of the median. B). Giemsa thin smears of P. falciparum. As compared to
untreated parasites, parasites treated with ADEP4 at 10 ug/ml lagged behind in the parasite cycle during the blood stage. When they were supposed to be trophozoites, they were rings, when they were supposed to be schizonts, they were trophozoites and they only get to finish their first cycle (ADEP4 Hour 72), when the untreated parasites are already in the middle of their second cycle (Untreated Hour 72).

**Figure 2** Effects of ADEPS on the biogenesis of the apicoplast during the first (A) and second cycle (B). As compared to untreated parasites, ADEP1 had no effect on the apicoplast biogenesis. ADEP4 however inhibited the biogenesis of the apicoplast. **Key:** DIC: Cells, Apicoplast: apicoplast anti-ACP primary antibodies stained with Alexa 546 secondary antibodies, DNA: Nucleus stained with Hoeschst, Merge: Combination of Alexa 548 and Hoeschst

**Figure 3** Growth inhibition assay of ADEP4 on *P. falciparum* under IPP provision. A). Parasites supplemented with IPP were still affected by ADEP4 at 10 µg/ml (ADEP4 10 IPP) and 30 µg/ml (ADEP4 30 IPP) as compared to control treated with DMSO and IPP. Error bars represent standard deviation of the median. B). Giemsa thin smears of *P. falciparum*. As compared to untreated parasites, parasites treated with ADEP4 at 10 ug/ml under exogenous IPP provision lagged much behind in the parasite cycle during the blood stage. They only get to finish their first cycle (ADEP4+IPP Hour 96), when the untreated parasites are already in their third cycle (Untreated Hour 96).

**Figure 4** Growth inhibition assay of ADEP4 on *P. falciparum* without the apicoplast under IPP provision. A). As compared to control, Clindamycin inhibited the apicoplast biogenesis towards the end of the second cycle. B). However, these parasites survived on provision with IPP (Clindamycin + IPP). C). These Parasites without the apicoplast but maintained under a continuous IPP supply were still
affected by ADEP4 at 10 µg/ml (IPP ADEP 10) and 30 µg/ml (IPP ADEP 30) as compared to the control treated with DMSO and IPP (IPP DMSO). P values represent Kruskal wallis rank test of the medians and error bars represents standard deviation of the median.
A

Parasite (%)

Time (Hour)

B

<table>
<thead>
<tr>
<th>Untreated</th>
<th>ADEP4+IPP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hour 24</strong></td>
<td>Ring</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>Trophozoite</td>
</tr>
<tr>
<td>Schizont</td>
<td>Trophozoite</td>
</tr>
<tr>
<td><strong>Hour 42</strong></td>
<td>Ring</td>
</tr>
<tr>
<td>Ring</td>
<td>Trophozoite</td>
</tr>
<tr>
<td><strong>Hour 48</strong></td>
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</tr>
<tr>
<td>Ring</td>
<td>Trophozoite</td>
</tr>
<tr>
<td><strong>Hour 72</strong></td>
<td>Trophozoite</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>Trophozoite</td>
</tr>
<tr>
<td><strong>Hour 96</strong></td>
<td>Ring</td>
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<tr>
<td>Ring</td>
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</tbody>
</table>

Legend:
- Control
- ADEP4-10 IPP
- ADEP4-30 IPP