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The apicoplast as a target for new interventions against malaria: further investigations towards a whole organism vaccination

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Content

List of abbreviations

(Pb)CSP	(*Plasmodium berghei*) circumsporozoite protein
µl	microliter
µM	micromolar (10^{-6})
µm	micrometre (10^{-6})
1st	First
2nd	Second
3rd	Third
Ap-	Apicoplast free
BSA	Bovine serum albumin
CAS	Chemically attenuated sporozoites
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
E64	trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane
EDTA	Ethylenediaminetetraacetate
e.g.	*Exempli gratia*
FCS	Fetal calf serum
GAS	Genetically attenuated sporozoites
h	hour
HBsAg	Hepatitis B surface antigen
HBSS	Hank's balanced salt solution
HEPES	hydroxyethylpiperazineethanesulfonic acid
hNTCP	Human sodium taurocholate cotransporting polypeptide
HSP70	Heat shock protein 70
i.e.	*Id est*
i.v.	Intravenous

IC50 / IC99	Inhibitory concentration 50/99%
IPP	Isopentenyl pyrophosphate
ITN	Insecticidal treated bed net
LSA-1	Liver specific antigen 1
ml	Millilitre (10^{-3})
mm	Millimetre (10^{-3})
nM	Nano molar (10^{-9})
NMRI	Naval Medical Research Institute
P.	*Plasmodium*
p.i.	*Post infectionem*
PbACP	*Plasmodium berghei* acyl carrier protein
PBS	Phosphate buffered saline
PFA	paraformaldehyde
RAS	Radiation attenuated sporozoites
RMPI medium	Roswell park memorial institute medium
Rpm	Rounds per minute
Spp.	*species pluralis*
WHO	World health organisation

List of figures

List of tables

1 Introduction

This study comprises two experimental approaches.

1. Further investigation towards a whole-organism vaccine against malaria by inhibiting the prokaryotic organelle within the malaria parasite using bacteriostatic antibiotics.
2. Effectiveness of drugs conjugated to the hepatitis B virus derived surface PreS protein. Eventually regarding liver specific delivery for the usage in whole organism vaccinations as well as an extended application possibility of these drugs.

1.1 Epidemiology of malaria and its impact on global health

Although malaria can be cured easily and both incidence and mortality are declining due to improved access to medical care and interventions, it still is a great global health burden: every two minutes a child dies of malaria - 720 of the youngest every day (WHO, 2017).

The world health organisation (WHO) declares in their annual report of 2018: 87 countries suffer from malaria transmission – over 3 billion people live at risk to be infected. In 2017, around 219 million cases were reported while the disease cost the life of 435.000 people, 61% of them children aged under 5 years (WHO, 2018).

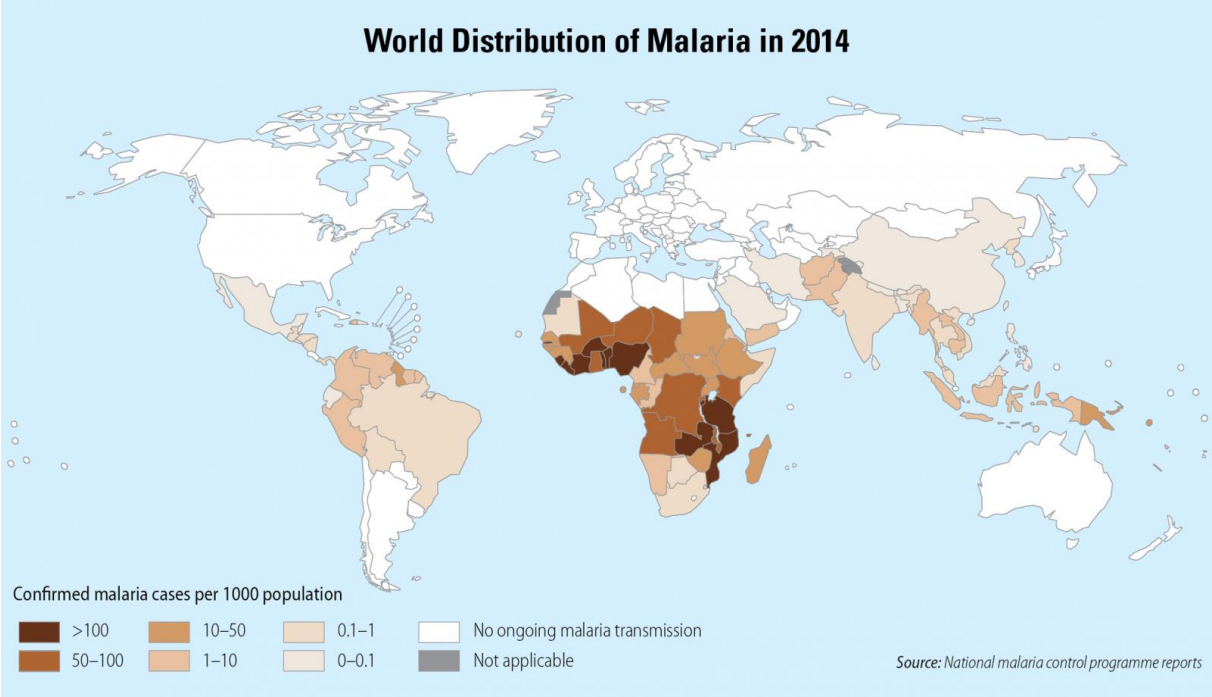


Figure 1. World distribution of malaria in 2014 (WHO, 2014). The majority of malaria cases occur in sub-Saharan Africa while South America and parts of Asia also suffer from the disease.

Malaria occurs in the tropics and subtropics while mainly sub-Saharan Africa is affected, with 92% of reported cases in 2017 occurred in the WHO African, 5% in the Southeast Asian and 2% in the Mediterranean region (WHO, 2018).

1.2 Malaria parasites and life cycle

Malaria is caused by a unicellular eukaryotic endoparasite. It uses the human being as an intermediate host plus the female *Anopheles* mosquito as both, a vector and host, to fulfil its complex life cycle.

1.2.1 Human pathogen *Plasmodium* species

Malaria-causing parasites belong to the genus *Plasmodium*, which includes about 200 species. Amongst them are five human pathogen parasites, namely *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi.*

Infections with these five human malaria parasites are associated with distinct clinical manifestations. These species also display different geographical distributions.

P. vivax occurs to 83% outside of Africa where it is the most common cause of malaria (WHO, 2016). It uses the Duffy-antigen on the host's erythrocytes to enter them. The loss of this antigen is a widespread natural immunity on the African continent which results in a lower burden of *P. vivax* infections there (Culleton et al., 2008).

P. falciparum is the dominant malaria parasite in Africa. It claims the majority (99%) of lives while 90% of all deaths occur on the African continent.

In this introduction, I focus on *P. falciparum* being the main human health threat. However, the data was partly collected using *P. berghei*, a rodent malaria parasite, as a model system. Since the aim of the study concerns the biology of *Plasmodium* species in general, this model represents a safe, well-established and suitable method to undertake these experiments.

1.2.2 Life cycle of human pathogen *Plasmodium* parasites

To pursue its transmission, the *Plasmodium* parasite depends on the female *Anopheles* mosquito as a vector and host plus a mammalian intermediate host.

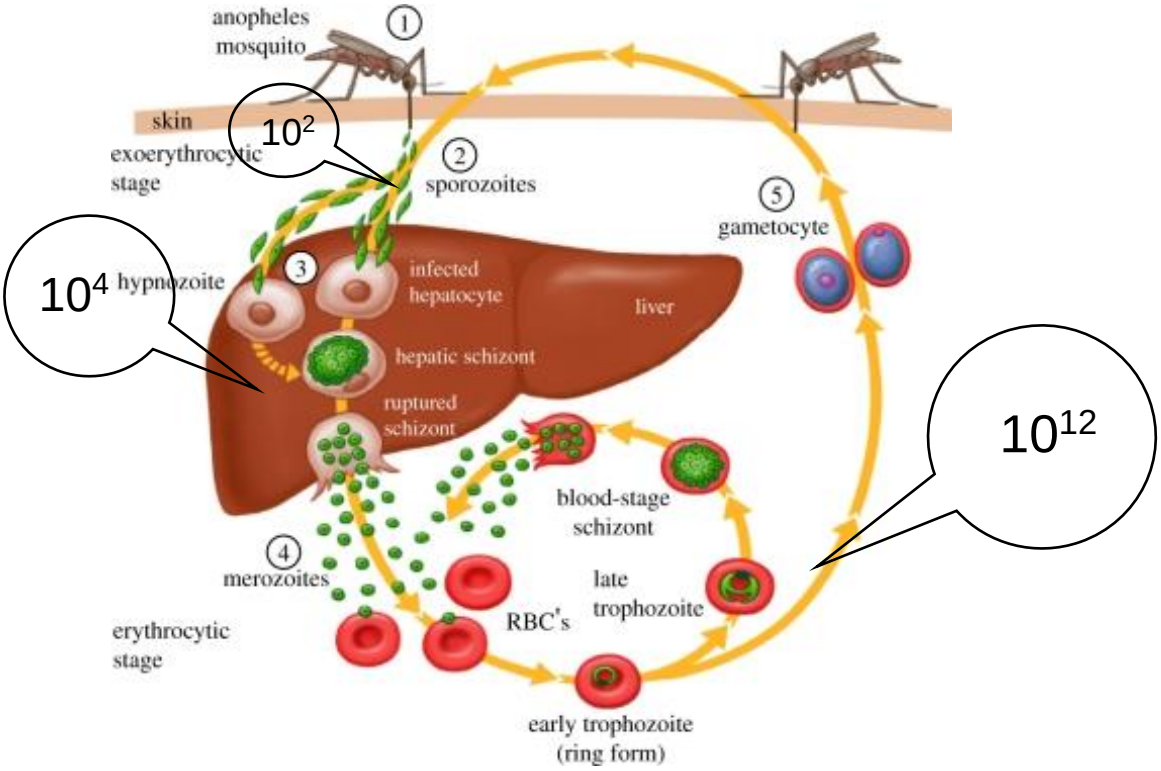


Figure 2. *Plasmodium* life cycle; modified after Hill (Hill, 2011). When an infected mosquito bites a mammalian host, up to 100 so-called sporozoites are delivered into the skin. They reach the liver, multiply, break out and invade erythrocytes. Only the massive replication of blood stages causes the typical flu-like symptoms of malaria like chills, headache, fever and fatigue.

Exo-erythrocytic stage

During a blood meal of the female *Anopheles* mosquito, up to a few hundred sporozoites are deposed in the skin tissue of the mammalian host. Sporozoites actively move and penetrate into local blood capillaries and from there, are passively delivered via the blood circulation to the liver (Medica and Sinnis, 2005, Amino *et al*., 2006).

In the liver, sporozoites glide inside the liver sinusoids along the hepatocytes and finally, invade hepatocytes after passing through resident Kupffer cells. Even after reaching a hepatocyte, sporozoites continue passing through several cells until settling down in a suitable cell and start forming a parasitophorous vacuole for further development (Pradel and Frevert, 2001, Frevert et al., 2005, Mota et al., 2001). Subsequently, the sporozoites differentiate and undergo schizogony:

they asexually build up a polyploid cell (schizont) by DNA replication. Nuclear division takes place only after multiple rounds of DNA replication (Striepen et al., 2007, Gerald et al., 2011). Eventually, daughter cells are build up: out of one single sporozoite up to 10^4 invasive blood stages, so-called merozoites, are formed (Sturm et al., 2009). Thereupon, merozoites emerge from the liver via a vesicle (merosome). These vesicles consist of 100-200 merozoites and bud off the detached host cell, probably allowing the parasite to hide from the host's immune system (Sturm et al., 2006, Baer et al., 2007). These merosomes reach the bloodstream and are transported to the capillaries of the lung, where they egress, hence releasing infectious merozoites (Baer et al., 2007). In the case of *P. falciparum,* the asymptomatic liver stage can take up to 8 days.

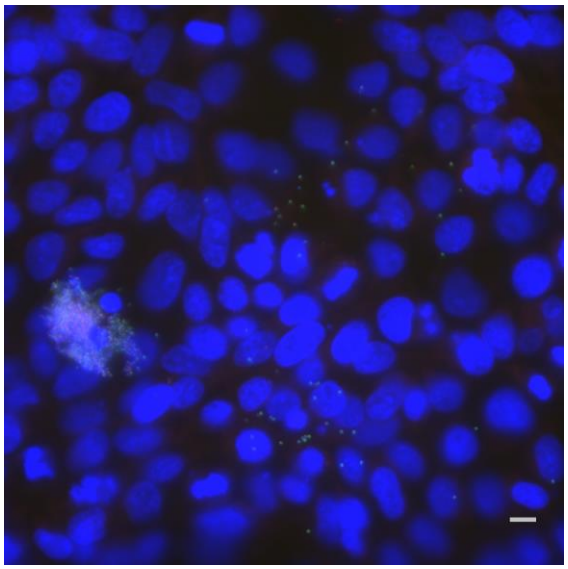


Figure 3. Ruptured merosome of *in vitro* cultured *P. berghei* in Huh7 cells. Representative micrograph (scale bar: 10 µm). Hoechst (blue) was applied for DNA staining, anti-HSP70 antibody to stain the parasite itself, appears green and anti-acyl carrier protein (ACP) antibodies to stain the apicoplast organelle inside the parasite (red). Note the several single merozoites emerged out of the vesicle. See experimental details further below.

Intra-erythrocytic stage

After their release, merozoites rapidly detach to and invade red blood cells, thought to minimise antigen stimulation of the host's immune system. Crucial for this invasion of host cells is the apical complex, containing a collection of specialised subcellular structures within the parasite. This complex is also present in other human pathogen parasites like *Toxoplasma gondii* or *Cryptosporidium parvum.* Indeed, the name of the phylum *Apicomplexa* stems from this unique structure (Cowman and Crabb, 2006, Arisue and Hashimoto, 2015).

After invasion, *Plasmodium falciparum* parasites undergo several replication cycles with a duration of 48 hours each, passing through different, microscopically distinguishable stages of development (ring stage – trophozoite stage – schizont stage – merozoites). Each cycle produces around 10-20 progeny, rapidly yielding enormous parasite loads of up to 10^{12} parasites.

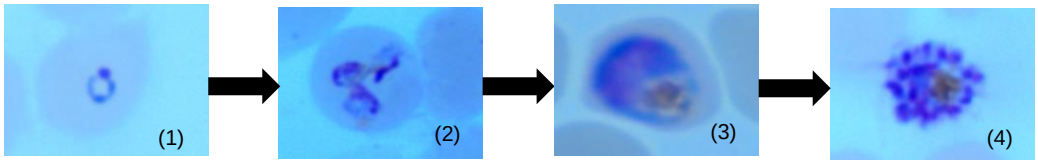


Figure 4. Developmental forms of blood stage parasites. During replication in erythrocytes, parasites undergo microscopically distinguishable differentiation. They turn from the first observed form, the ring stage (1) to trophozoites (early, 2 and late, 3) and subsequently to schizonts (4). Within schizonts, new daughter cells, so-called merozoites are formed (4). Finally, merozoites egress from the host cell (not shown) and re-invade new erythrocytes; hence start a new replication cycle.

Whereas *P. falciparum's* multiplication is not synchronised, the other human pathogen *Plasmodium* parasites multiply in a synchronised pattern, resulting in individual courses of disease defined by specific alternation of fever peaks and fever afebrile episodes. However, only *P. falciparum* infections can lead to severe malaria, characterised by coma, metabolic acidosis, severe anaemia, hypoglycaemia and renal failure. Cerebral malaria (with deep, unarousable coma

as the leading symptom) is thought to be due to micro-embolic infarcts owing to parasite-induced cytoadherence and sequestration of infected red blood cells in the deep vasculature of the brain (Miller et al., 2002, Alister et al., 2012). This serious scenario is likely associated with high case fatality ratio or in survivors, with disabling long-term health issues like neurological disorders.During multiplication of the parasites within erythrocytes, a variable proportion of ring stages start differentiating into sexual stages, called gametocytes. These female or male parasite stages are again taken up by female *Anopheles* mosquitos during a blood-sucking bite. Inside the mosquito's midgut, exflagellated male gametocytes are able to fertilise female gametocytes and then turn into zygotes.

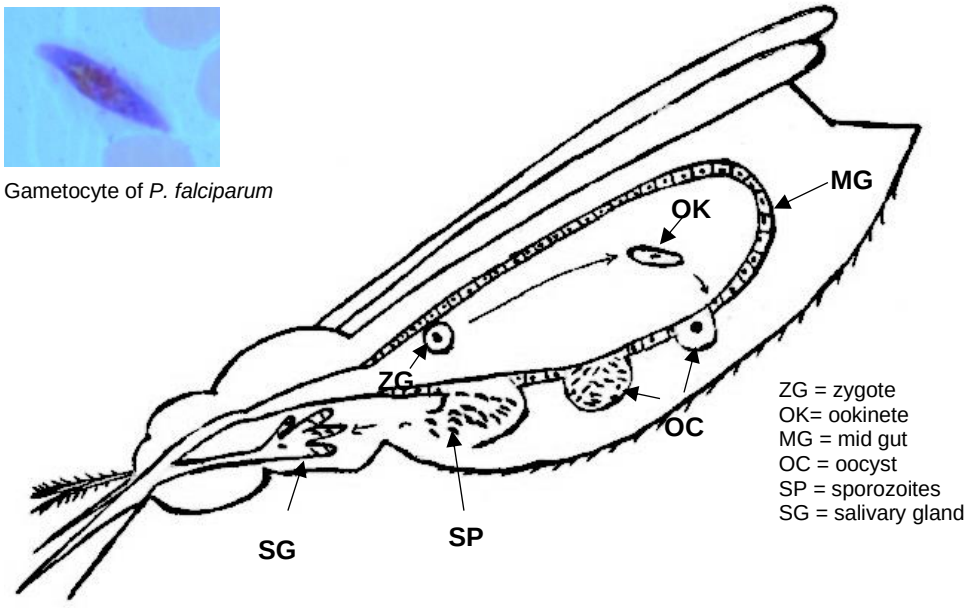


Figure 5. Mosquito stage of the *Plasmodium* life cycle. Incorporated female and male gametocytes fertilise inside the midgut of female *Anopheles* mosquitos. They form diploid zygotes and further differentiate develop into ookinetes and meiosis begins. Ookinetes are actively able to access the midgut epithelia. They mature to oocysts producing motile sporozoites (haploid) which invade the salivary gland of the mosquito.

Inside the vector's or rather host's midgut, the zygote differentiates into an actively moving ookinete and at the same time, initiates sexual replication (meiosis). After penetration of the midgut epithelium, the ookinete transforms into

an oocyst and completes meiosis. Asexual replication sets in, producing thousands of haploid, actively moving sporozoites via schizogony. Once sporozoites reach the salivary gland, female *Anopheles* mosquitoes are infectious for around 2-3 weeks, hence the transmission cycle starts again (Mueller et al., 2010).

1.3 The apicoplast of *Plasmodium spp*.

As mentioned above, *Plasmodium* parasites belong to the phylum *Apicomplexa*. Not only do they share an eponymous apical complex, but most additionally share a mutual organelle, called apicoplast.

This prokaryotic organelle derives from a chloroplast (which itself has its origin in a cyanobacterium) and is surrounded by four membranes. These findings suggest that the apicoplast entered the parasite via secondary endosymbiosis.

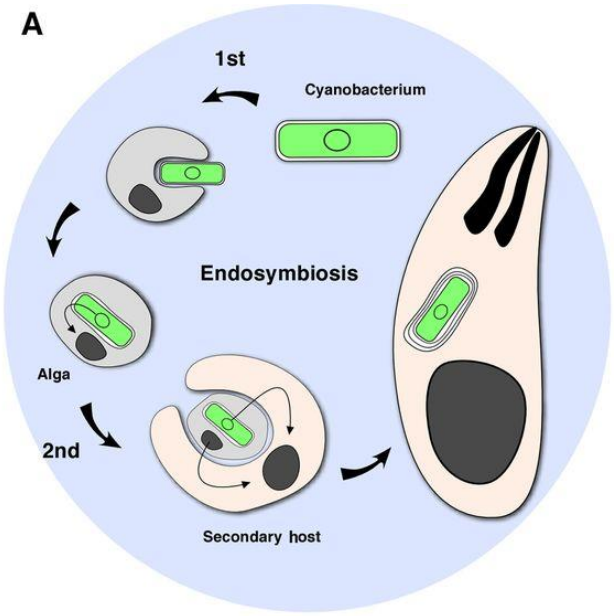


Figure 6. Secondary endosymbiosis of the apicoplast (Striepen, 2011). In the beginning, a cyanobacterium was engulfed by a eukaryote cell. Thus, an alga arose, which itself merged with another eukaryotic host cell to form the common ancestor of apicomplexan parasites.

During the first endosymbiosis, a cyanobacterium was engulfed i.e., via phagocytosis, by a eukaryotic cell (probably a biciliate protozoan) around 1.5 billion years ago – together forming the ancestor of all green algae and plants, red algae and glaucophytes. These cells were now able to generate energy through photosynthesis inside the cyanobacterium-derived organelle (chloroplast). The available genetic evidence then points to a secondary endosymbiosis event: a red alga was taken up in by another eukaryotic cell, giving rise to the phylum *Apicomplexa*. While apicomplexan parasites such as *Plasmodium* still harbour the prokaryotic plastid, it has lost its photosynthetic capacity (Lim and McFadden, 2010).

1.3.1 Function of the apicoplast in *Plasmodium*

The apicoplast still contains a 35 kb DNA genome coding for a few tRNAs and housekeeping proteins. However, most of the proteins required for its function and replication are encoded in the parasite's nucleus and imported into the organelle. Because of the evolutionary history of the apicoplast, these proteins are either of prokaryotic origin (following the transfer of genes from the endosymbiont to the host cell nucleus) or of eukaryotic origin. Thus, the few biochemical pathways inside the apicoplast consist of a mosaic of enzymes of different evolutionary origin (Martin and Herrmann, 1998, Waller et al., 1998).

Still, the parasite is not able to survive without the remnant, indeed the apicoplast harbours essential metabolic pathways on which the parasite depends throughout different stages during its life cycle (Ralph et al., 2004). Firstly, the heme biosynthesis pathway, which is essential during development in the mosquito and the liver is partially located inside the apicoplast (Ke et al., 2014, Rathnapala et al., 2017). Secondly, the organelle harbours the type II fatty acid synthesis which is crucial in the late liver stage development of the parasite (Yu et al., 2008). Additionally, during blood stage infection and especially, egress from the infected red blood cells, isopentenyl pyrophosphate (IPP) production within the plastid plays a key role (own data). Interestingly, supplementing solely IPP can fully compensate for the loss of the whole organelle in blood stage parasites

(Yeh and DeRisi, 2011). Hence, this relict plastid is essential for parasite survival (He et al., 2001).

1.3.2 The apicoplast of *Plasmodium* as a target

Every parasite hosts only one apicoplast which must be inherited vertically to every daughter cell during asexual replication in liver, blood and mosquito stages. Biogenesis of the apicoplast can be observed during schizogony as a branching structure, followed by fission at the end of a replication cycle and distribution into every descendant in a 1:1 fashion (Waller and McFadden, 2005).

Direct inhibition of metabolic pathways located in the apicoplast, in other words targeting non-housekeeping processes, e.g. blocking of IPP production in blood stages via fosmidomycin induces an immediate parasiticidal effect (Missinou et al., 2002).

On the other hand, using housekeeping functions of the apicoplast in blood stage parasites as a target results in a “delayed death”. This means that parasite proliferation is arrested at the end of the second cycle of replication in human erythrocytes, 96 hours after the start of the first cycle (Ramya et al., 2007). This can be achieved by applying bacteriostatic antibiotics. Bacteriostatic antibiotics stop bacteria in their metabolism but do not kill them, in contrast to bactericidal antibiotics. For example, bacteriostatic antibiotics like clindamycin, tetracycline or azithromycin bind and block prokaryotic ribosomes, targeting the apicoplast’s own translational machinery (Wilson et al., 1996). This seems to impede the organelle’s inheritance but does not kill it immediately, and thereby induce delayed death in blood stage parasites (Burkhardt et al., 2007, Dahl et al., 2006, Goodman et al., 2016).

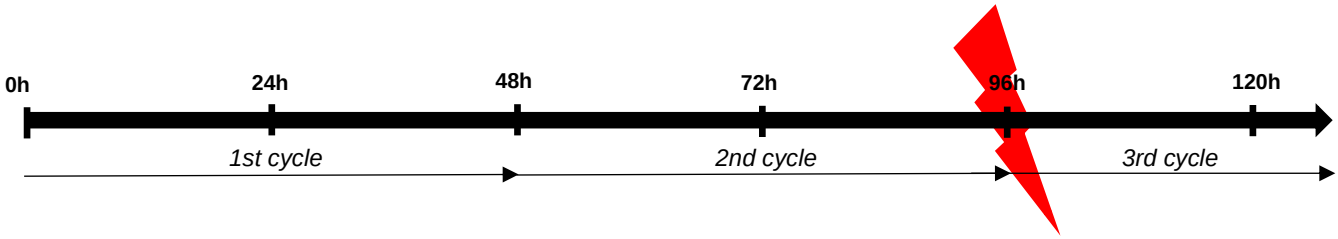


Figure 7. Targeting housekeeping functions of the apicoplast leads to a delayed death of blood stage parasites after 96h of initial erythrocyte invasion.

Since the killing of blood stage parasites is delayed, these antibiotics cannot be used alone for the treatment of malaria.

However use of these drugs, shown for azithromycin, against the preceding life cycle stage of malaria parasites in the liver (characterised by a single round of replication), leads to the release of non-infectious merozoites, hence preventing the pathogenic blood stage proliferation (Prudencio et al., 2006, Friesen et al., 2010). The reason for the difference in the killing kinetics between liver stage and blood stage is not clear. Possibly, the immediate effect of antibiotics against liver stages could be related to the elevated requirement for housekeeping proteins caused by the massive replication in the liver stage (producing tens of thousands of progeny). Disruption of apicoplast biogenesis and thus, inhibiting *de novo* fatty acid synthesis would be expected to lead to parasite arrest at the late liver stage. Indeed, the pharmacologically induced anti-liver stage effect phenocopies the type II fatty acid knockouts (Yu et al., 2008).

The essential role of the apicoplast for the survival of blood and liver stages offers a great opportunity as drug target for interventions and prophylaxis against malaria, including regarding emerging resistance to current first-line treatments (Mukherjee and Sadhukhan, 2016).

Additionally, since liver stage infection depends on fewer parasites than blood stage infections, representing a smaller parasite burden and furthermore an asymptomatic infection state - this “bottleneck” of the parasite’s life cycle is a promising starting point for prophylactic interventions: inducing sterile immunity by antigen presentation to the host’s immune system without causing malaria symptoms.

1.4 Naturally acquired immunity against *P. falciparum* malaria

Residents in endemic malaria areas develop a partial immunity against *P. falciparum* infections (also named ‘semi-immunity’). Immunity that shields against severe malaria can be acquired after only a few malaria episodes and is further

enhanced after multiple re-infections, preventing also uncomplicated disease episodes (Gupta et al., 1999). However, immunity that limits parasite proliferation develops much slower and there is controversy over providing full sterile protection does not seem to provide full sterile protection against re-infection (K. Marsh, 2006, Tran et al., 2013, Lell et al., 2018).

1.5 Current prevention strategies against malaria

The best protection against malaria is the prevention of infection. Several intervention strategies are currently being used for malaria control.

1.5.1 Implemented prevention strategies against malaria

Several approaches of malaria prevention have been successfully implemented worldwide, resulting in declining numbers of cases and deaths: in the five year period from 2010 to 2015, a decrease of 21% of reported cases and 29% of deaths was achieved (WHO, 2017).

First of all, personal protection measures such as insecticide-treated bed nets (in 2015, 57% of the population living at risk sleeping under ITN) or indoor residual spraying for vector control play the main role in preventing malaria: from 2001 to 2015 69% of cases have been prevented thanks to ITNs (Bhatt et al., 2015). Still, in 2015 43% of people living at risk were not protected with either of the options. Secondly, fast access to diagnosis, treatment and medical care is important. Thirdly, various intermittent preventive treatment (IPT) schemes with anti-malarial drugs are being deployed for averting malaria in the most vulnerable population groups: pregnant women and children. IPTs have been shown to be effective but coverage has to be expanded further (WHO, 2017).

Probably, the most promising long-lasting way to eliminate malaria is a vaccination. This has been demonstrated for other diseases, showcased by the success of the smallpox virus vaccine (Henderson, 1987).

The first vaccine candidate against *P. falciparum* malaria, RTS,S/AS01 is a recombinant vaccine consisting of T-cell and B-cell epitopes of the circumsporozoite protein of *P. falciparum* fused to the hepatitis B surface antigen. RTS,S has received a positive opinion by the EMEA but is not yet available on the market. It will be further studied in a large implementation phase IV trial in three sub-Saharan African countries (WHO press release 17.11.2016). The vaccine provides limited protection (17% efficacy against infection after 5 years) in children 5-17 months old (White et al., 2015). Although RTS,S shows that a malaria vaccine can be developed, further development of other promising approaches, e.g. whole-organism vaccine protocols, is needed to attain a safe, affordable and perhaps even more potent vaccine.

1.6 Whole organism vaccinations

Considerable progress has been made with whole-parasite vaccine protocols. These protocols rely on the inoculation of volunteers with either live or attenuated parasites, either as sporozoites or as blood stages. For experimental vaccine protocols, sporozoites can be inoculated via bites from infected *An. stephensi* mosquitoes or via intramuscular or intravenous injection of aseptic, cryo-preserved sporozoites isolated from sterilely raised *An. stephensi* mosquitoes. Wildtype or attenuated sporozoites that are able to infect hepatocytes provide antigen stimulation of the host's immune Infections are arrested either during the liver stage or during the first intra-erythrocytic cycle, preventing potentially life-threatening pathogenic blood stage proliferation. These protocols can induce sterile immunity against challenge with the same strain but provide only limited strain-transcending protection (Meta Roestenberg et al., 2009, Mordmuller et al., 2017, Walk et al., 2017).

Regarding the potency of protection, hepatocyte invasion and subsequent parasite maturation within the liver cell correlates with robust immunity. Protection depends on the priming of CD8+ T-cells producing interferon γ (W. Weiss, 1988, Schofield et al., 1987, Jobe et al., 2007, Friesen et al., 2010). This accounts especially regarding long term protection (Nganou-Makamdop et al., 2012b).

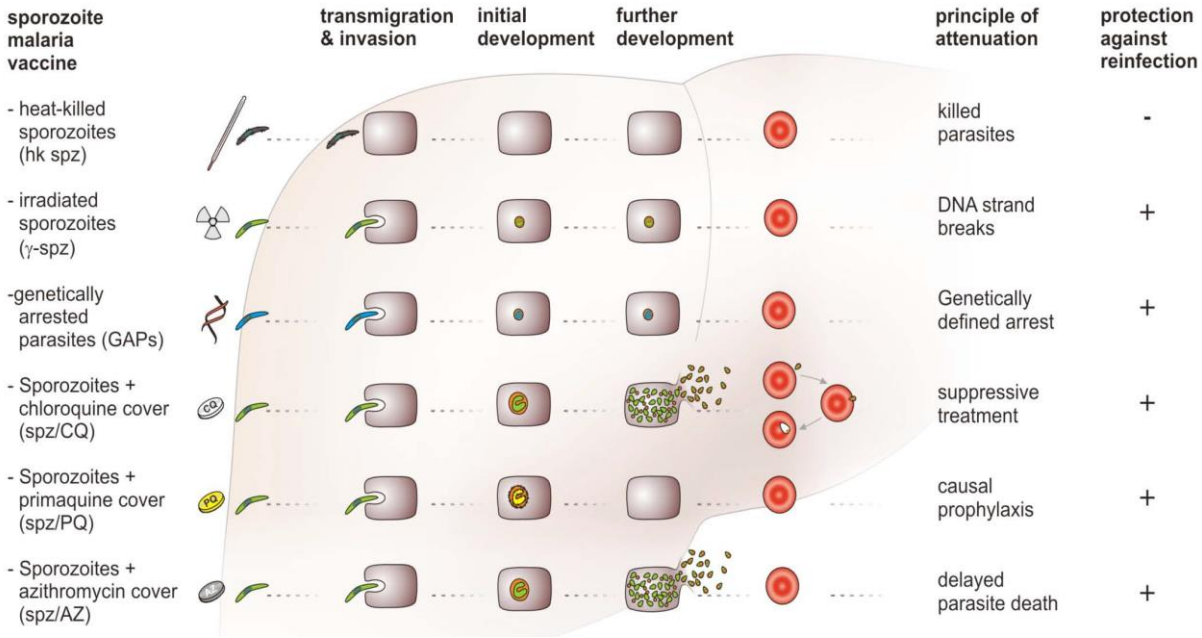


Figure 8. Overview of whole organism vaccination approaches (Matuschewski et al., 2014). Several strategies to attenuate *Plasmodium* sporozoites followed by inoculation induce sterile protection against reinfection with the same strain while differences in the parasites' maturation inside hepatocytes exist.

First trials using heat-killed sporozoites showed the need of metabolically active and invasion competent parasites. The vaccination did not induce long term sterile protection (Hafalla et al., 2006, Alger and Harant, 1976).

This limitation was overcome by the use of radiation, leading to DNA strand breaks of sporozoites' genome (RAS). Heterologous protection was induced by immunisation with bites from thousands of radiation attenuated mosquitos harbouring the parasite (Hoffman et al., 2002).

First shown in a murine model by Mueller *et al*., genetically attenuated parasites (GAS) can be used as an effective approach for inducing sterilising immunity. They used knock-out lines lacking an essential gene important in early liver stage development in *P. berghei* parasites (uis-3) (Mueller et al., 2005).

Importantly, administration of chemoprophylaxis during inoculation of live, fully infectious sporozoites (CVac) using chloroquine was shown to induce fully sterilising immunity in humans (Mordmuller et al., 2017, Meta Roestenberg et al.,

2009). Prophylaxis with primaquine also resulted in notable, though incomplete, protection (Fryauff et al., 1997).

The simultaneous usage of azithromycin, a macrolide antibiotic, during inoculation of sporozoites revealed a protective immunity against reinfection in rodents (Friesen et al., 2010).

1.6.1 Advantages and disadvantages of different whole organism vaccination approaches

There are several aspects which have to be taken into consideration regarding whole organism vaccinations.

To begin with, logistical problems have to be faced. Due to ethical, safety and controlling concerns, vaccination using easily accessible infected mosquitos would not receive medical approval due to high infection risk in the manufacturing process, coinfection with other pathogens, variable infectivity etc. However, it was shown that transportable cryopreserved sporozoites are able to induce malaria infection, hence can be used for a chemically attenuated parasite vaccine (Roestenberg et al., 2013). Yet, since intravenous inoculation of the vaccination is followed by better protection against reinfection well trained local medical professionals are required to applicate and supervise the immunisation (Parmar et al., 2016). This is an important aspect especially in African countries with poor medical supply.

An important limitation of these approaches is that they induce primarily sterile protection against the same strain, i.e. homologous immunity. Due to the parasite's huge antigen diversity, this is not a predictor for a sterile protection after natural exposure. On the other hand, there have been also reports about cross-stage and even cross-species immunity induced by especially pre-erythrocytic whole organism vaccinations in mice (Douradinha et al., 2008, Butler et al., 2011, Inoue et al., 2012). Hence, antigen diversity between different parasite strains and variety in prevalence of strains in various regions have to be considered for further investigations of a whole parasite vaccination. For example, characterisation of the most common parasites in individual regions and specific vaccination combinations.

There are some disadvantages taking a closer look at the different approaches:

A key concern with radiation-attenuated sporozoites involves the aspect of safety. When sporozoites are over-irradiated no protection is achieved whereas inoculation of under-irradiated sporozoites can result in incomplete arrest during the liver stage and thus, life-threatening blood stage infections after vaccination (Silvie et al., 2002). Regarding genetically attenuated parasites, similar concerns over the manufacturing process were raised. Faulty gene knock out led to clinical malaria in subjects after immunisation (Spring et al., 2013). Since manufacturing of chemically attenuated parasites does not depend on the genetic stability and phenotype of knock-out lines or DNA breaks, there have been no concerns regarding production processes.

Another important aspect is the timepoint of parasite arrest. Especially the late liver stage arrest is predominant regarding the induction of protective immunity (Nganou-Makamdop and Sauerwein, 2013, Butler et al., 2011). Chemically attenuated parasites show this late stage arrest, whereas most of genetically or radiation attenuated parasites are stopped earlier in liver stage development: Previous research showed that late-attenuated parasites can induce a better protection on a per-injected sporozoite basis against challenge than irradiated sporozoites (Friesen and Matuschewski, 2011).

However, as shown in Figure 8, injection of chloroquine attenuated parasites is followed by maturation of the parasite within the liver and even erythrocyte invasion, hence can cause a mild malaria infection. In contrast, certain approaches using drug cover of parasites with antibiotics, e.g. azithromycin, lead to a late liver stage arrest, hence parasites are able to undergo full liver stage development and can even emerge from the liver as merozoites but are unable to invade erythrocytes and do not cause malaria symptoms (Matuschewski et al., 2014).

Additionally, the immunisation approach using chloroquine shows less protection than attenuation with azithromycin in mice. This might be due to modulation of the immune system by blood stage parasites and presents one more argument in favour of late liver stage arrest (Friesen and Matuschewski, 2011). Apart from

this, although chloroquine is a well-known anti-malarial drug, there is one main disadvantage: resistance especially in African countries (Price et al., 2014). Whereas, there are rarely studies concerning resistance of *Plasmodium* parasites to antibiotics like clindamycin resistance in the Amazonas region or induced *in vitro* resistance to azithromycin (Dharia et al., 2010, Sidhu et al., 2007). This has to be taken into account considering individual vaccination combinations for specific regions.

In this study, I concentrated on the new macrolide antibiotic solithromycin and its potential in a whole organism vaccination approach using chemically attenuated parasites.

1.6.2 Characterisation of solithromycin

Solithromycin is a new fluoroketolid antibiotic, a next-generation macrolide. Ketolids are derivatives of the macrolide erythromycin supplemented with a ketone group which enables them to bind and inhibit at two sides of the bacterial ribosome's 50s subunit, hence inhibit protein translation even more effective. This additional binding side leads to a broader efficacy (Fernandes et al., 2016).

To solithromycin, the first fluoroketolid, fluorine is added. This results in a third binding site at prokaryotic ribosomes, hence a higher affinity to ribosomes as well as a potency to act efficiently against macrolide and even ketolide resistant bacteria (Llano-Sotelo et al., 2010, Fernandes et al., 2016). In comparison to its predecessor, the well-known ketolide telithromycin, solithromycin does not possess a pyridine sidechain which is known to inhibit nicotinic acetylcholine receptors entailing severe adverse effects like visual disturbance or liver toxicity (Fernandes et al., 2016).

The compound is to date in phase 3 clinical development for treatment of community acquired bacterial pneumonia. Previous studies showed a safe use in patients including children and pregnant women and people with liver impairment (Barrera et al., 2016, Fernandes et al., 2016, Keelan et al., 2016, Jamieson et al., 2015). However, it was not yet approved by the FDA, which recently expressed

concerns about the lack of investigations and data regarding a possible liver toxicity of the compound (Abdulla, 2016).

Regarding antimalarial potential, solithromycin induces delayed death in both, *P. falciparum* and *P. berghei* blood stages. Moreover, it is more potent than azithromycin against multidrug resistant, including azithromycin resistant, lab strains of *P. falciparum in vitro* and shows a higher antimalarial activity against *P. berghei in vivo* compared to azithromycin (Wittlin et al., 2012). Furthermore, higher intracellular concentrations than with other macrolides have been achieved with this compound (Lemaire et al., 2009). Additionally, malnutrition, which is common in African countries, showed no effect on its bioavailability (Still et al., 2011).

1.7 Usage of the Hepatitis B virus' PreS surface protein to gain liver specific delivery

The PreS protein is well known from the hepatitis B virus (HBV).

The HBV is a highly selective hepatotropic virus. It expresses a hepatitis B surface antigen (HbsAg) consisting of different proteins. The large surface antigen consists of the preS1, preS2 and S domain. Consisting of solely the preS2 and S domain, it is called middle surface antigen. Consequently, S equals the small surface antigen (Stibbe and Gerlich, 1983).

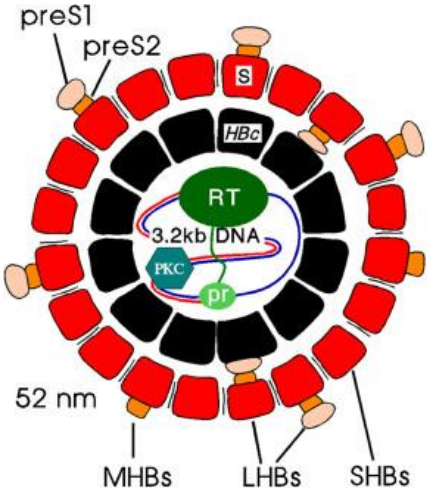


Figure 9. Structure of the hepatitis B virus (Gerlich, 2013). Note the different surface proteins. LHBs= large hepatitis B surface protein, MHBs= middle hepatitis B surface protein, SHBs= small hepatitis B surface protein, are collectively the hepatitis B surface antigen.

The PreS1 domain of the large surface protein is the virus' key for entering the liver by binding to the sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane transporter on the hepatocyte surface (Yan et al., 2012, Meier et al., 2013).

1.7.1 Physiological function of the NTCP

Hepatocytes physiologically express a sodium taurocholate cotransporting polypeptide on their basolateral membrane for reuptake of conjugated and unconjugated bile acids from the portal vein within the enterohepatic circulation (Mita et al., 2006).

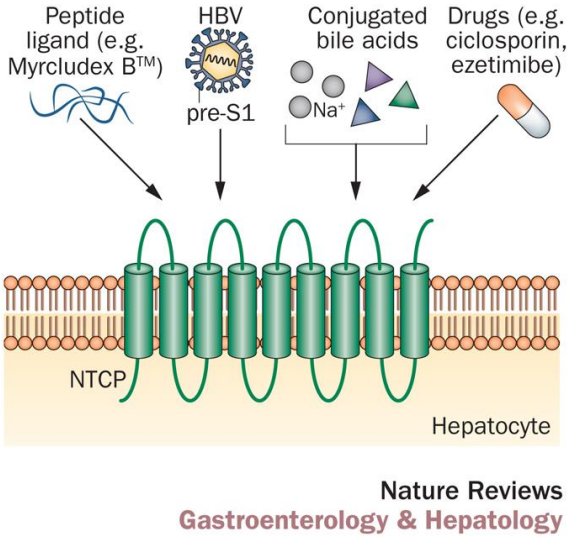


Figure 10. The NTCP of hepatocytes, its physiological function and targeting structures (Yuen and Lai, 2015). The sodium taurocholate cotransporting polypeptide is expressed on hepatocytes for bile acid reuptake but also serves as the functional receptor of the hepatitis B virus and iatrogenic interventions.

1.7.2 Therapeutic approaches

The identification of NTCP as the functional receptor for the hepatitis B virus opened several novel approaches to inhibit the virus' entry of the liver. Novel interventions include preventing the virus' access to the liver by blocking the receptor e.g. with ciclosporin or Myrcludex B, which is a derivative of the large surface protein of HBV (Yuen and Lai, 2015, Petersen et al., 2008, Watashi et al., 2014a).

This knowledge about the entry mechanism of HBV was also used by Zhang *et al*. to create a liver specific transportation. The conjugation of a lipopeptide derived from the PreS1 surface antigen and pegylated liposomes resulted in precise transport of conjugated drugs to hepatocytes. This approach showed a higher efficacy of these drugs within the liver parenchyma (Zhang et al., 2015). Lately, an approach using a conjugation of lipid modificated nanoparticles and myristoylated PreS-peptide also showed a specific binding to NTCP as well as initialization of molecules into hepatocytes (Witzigmann et al., 2019).

A specific transportation to the liver of drugs is an interesting approach for the usage of antibiotics in whole organism vaccinations. Peptide-conjugated drugs could be used as an inoculation additive and so be applied at the same time parasites are injected into the patient. Giving a single shot of this combination could result in the reduction of drug dose as well as reduction of systemic side effects of the antibiotic applied and an even stronger efficiency as well as improved patients' compliance.

Additionally, the usage of PreS1 for liver specific transportation could be used for already established anti-malarial drug therapies. For example, primaquine which cannot be applied as a treatment regarding special patient groups.

1.8 Primaquine and its adverse side effects

Primaquine is indispensable in malaria treatment. It is the only drug capable of eliminating liver hypnozoites of *P. vivax* and *P. ovale,* available since the 1950s (John et al., 2012). Additionally, *P. falciparum's* sexual blood stages (gametocytes) are sensitive to the drug, which results in a lower count of gametocytes, hence a reduced transmission of the parasite (Graves PM, 2014).

However, there are significant adverse side effects associated with this drug. It can induce haemolysis in people suffering from Glucose-6-phosphate-dehydrogenase deficiency (Clyde D.F., 1981). This is a big issue in sub-Saharan Africa because G6PDH deficiency represents a natural protection against severe Malaria and therefore is widespread in Malaria endemic areas (Mbanefo et al., 2017).

Primaquine is also contraindicated in pregnancy (Fernando et al., 2011). Pregnant women represent a special risk group regarding malaria infection: the women's immune system is complex modulated and therefore more prone to malaria infections (Szekeres-Bartho and Wegmann, 1996, Mor and Cardenas, 2010). Additionally, a special surface antigen is expressed in infected red blood cells (VARS2CSA) which binds to receptors expressed on the placental surface (chondroitin sulphate A). This sequestration can end in placental insufficiency

(Takem and D'Alessandro, 2013, Ismail et al., 2000). Women also suffer from undetected *P. vivax* rebounds during pregnancy (Yanow et al., 2016).

Because of these severe side effects, it would be preferable if primaquine could be delivered specifically to hepatocytes only.

1.9 Aim of the study

Despite being a treatable and preventable disease, malaria still has a huge impact on global health. There are several successfully implemented strategies to contain this disease and help people living at risk of infection. Additionally, as described above, the first anti-malarial vaccine candidate is on its way to approval but does not provide the efficacy wished for.

There still is the need to develop a safe, affordable and effective vaccine and eventually eliminate this disease worldwide.

The first part of this study pays attention to an antibiotic-based inhibition of the malaria parasite. I focused on the new antibiotic solithromycin and if it is a suitable candidate to attenuated malaria parasites regarding the usage in a whole organism vaccination protocol.

At a first step, solithromycin's mode of action on *P. falciparum* blood stage parasites was tested to identify its attenuation effect and target within the parasite. Secondly, solithromycin treated parasites were tested in a mouse model to verify the capability of parasite development in hepatocytes, hence immune system stimulation. However, the injection of merosomes which emerged from hepatocytes into mice was not followed by parasitemia, thus these noninfectious merosomes were not able to cause malaria symptoms.

As a second part, I focused on the potency of PreS1 conjugated drugs regarding liver specific delivery. In this study, I examined the effect of PreS1-conjugated primaquine, an anti-malarial drug.

PreS1 is a component of the hepatitis B virus' large surface antigen, crucial for hepatocyte invasion. This hepatotropic characteristics can be transferred to a drug by conjugation, thus reducing the specific systemic adverse side effects and dose. In this case, the usage of this conjugation is interesting regarding an application within a whole organism vaccination using antibiotics. As an injectable conjugation, it could be applied simultaneously with the parasites even in one single syringe. I studied the effect of PreS1-conjugated primaquine, whose effect on *Plasmodium* parasites are well-studied. The conjugation was tested on *P. berghei* liver stage parasites developed in hNTCP HuH7 cells.

2 Materials and Methods

2.1 Materials

Disposables

1.2µm Syringe filter	Sartorius
8 well chamber slides	Thermo scientific
Aluminium foil	Roth
Coverslips	Marienfeld
Cryovials	Greiner bio-one
Culture flasks T25 and T75	Greiner bio-one
Gloves Nitrile powder free	Abena
Insulin syringes	BD Discardit II
Microscope slide	Thermo Scientific, ground edges, frosted ends
Needles (27G, 23G, 30G)	BD Microlance
Pipette tips	Greiner bio-one
Sterile filter	Milipore, 0.22 µm, steritop
Stripettes	Sarstedt
Tubes 15ml, 50ml	Falcon, Sarstedt, greiner bio one
Tubes 1ml, 2ml	Eppendorf

Chemicals

0+ erythrocytes	Zentrum für Klinische Transfusionsmedizin, University Tübingen
Albumax II	Invitrogen
Anti-anti	Life technologies
Azithromycin	Pfizer
Bovine Serum Albumin	Roth

Disinfection	Descosept AF
DMEM medium	Life technologies
DMSO	gruessing
E64	Sigma Aldrich
FCS Us certified heat inactivated	Life technologies
Gentamicin	Invitrogen
Giemsa	Merck
HBSS	Life technologies
Human AB+ serum	Zentrum für Klinische Transfusionsmedizin, University Tübingen
Hypoxanthine	ccpro
Immersion oil	Immersol^{TM} W Zeiss
IPP	Sigma Aldrich
Macs columns	Miltenyi biotec
Methanol	Sigma- Aldrich
Nailpolish	Essence
Penicillin/streptomycin	Life technologies
PFA	Roth
Phosphate buffered Saline	Life technologies
PreS1-Primaquine	AG Urban, University of Heidelberg
Primaquine	Mpbio
RPMI 1640	Thermofisher Scientific
Solithromycin	Cempra
Sorbitol	Fluka Biochemika
Trypsin EDTA	Life technologies

Equipment

Climate chamber	Mytron

Centrifuge	Hettich Rotanta
Centrifuge 5415R	eppendorf
CO_2 incubator	Thermo scientific HERAcell 150i
Freezer -20°C, Fridge 5°C	Liebherr
Freezer -80°C	Heraus, thermo scientific
Haemocytometer	Labotec
Light microscope	Nikon Eclipse E200
Megafuge R 1.0	Heraus
Microscope Axioskop 40	Zeiss
Mosquito cages	BioQuip Products Inc
Pipette dispenser	Integra pipette boy
Pipettes	Eppendorf, Abimed
Scale	Mettler Toledo
Stereo microscope Stemi 2000C	Zeiss
Water bath	Bachofer
Work bench Hera safe	Thermo fisher
Zeiss Axio Observer Z.1	AG Schenke-Layland Dept.of Women's Health Research Institute for Women's Health, Tübingen

Antibodies

Alexa Fluor 488 (goat anti-mouse)	Invitrogen
Alexa Fluor 546 (goat anti-mouse)	Invitrogen
Alexa Fluor 546 (goat anti rabbit)	Invitrogen
Anti PbACP serum	eurogentec
Anti PbCSP	Hybridoma supernatant
Anti PbHSP70	Hybridoma supernatant
Hoechst 33342	Invitrogen

Biologicals

Anopheles stephensi	Max Plank Institute for Infection Biology, Berlin
Huh7 hepatoma cell line	AG Bartenschlager, University of Heidelberg
HuH7hNTCP cell line	AG Urban, Centre for Infectious Diseases, Molecular Virology, Heidelberg
NMRI mice	Janvier, France

Computer softwares

Fiji	ImageJ 1.51e
Prism 5.0	Graph Pad
Endnote X7.5	Thomson Reuter

2.2 Methods of *Plasmodium falciparum* 3D7 blood stage parasite culture

Plasmodium falciparum 3D7 parasites were cultured *in vitro* modified after Trager and Jensen (Trager and Jensen, 1976).

2.2.1 Blood culture conditions of *P. falciparum* 3D7 parasites

Parasites were cultured in T75 or T25 culture flasks. The complete medium contained RPMI 1640 (supplemented with L-Glutamine and 25nM HEPES), 10% Albumax II, 2% Human AB+ serum, 1% Hypoxanthine and 0.05% Gentamicin; 0+ erythrocytes at a haematocrit of 5% were supplemented, cultures were kept in an incubator at 37°C, 5% CO_2 and 5% O_2.

For observing growth and parasitaemia of the culture, thin blood smears were prepared by using circa 2 µl of the culture's erythrocytes and spreading them equally on a microscope slide.

Subsequently, the smear was fixed for 1 minute in 100% methanol and stained 15 minutes in 5-10% Giemsa solution. The slides were examined by light microscopy using a 100x objective and immersion oil.

Parasitaemia was kept at around 1-2% by changing medium and blood regularly.

2.2.2 *In vitro* drug assay

To test the potency of the drug at time point zero, particularly with regard to a possible inhibition of erythrocyte invasion, merozoites were isolated modified after Boyle *et al.* (Boyle et al., 2010b).

Untreated *Plasmodium falciparum* 3D7 blood cultures show a mixed picture of parasite's stages. To synchronise the parasites' development, the following procedure was undertaken:

Table 1. Experimental setup of *P. falciparum* 3D7 blood culture synchronisation.

Day of synchronization	Treatment	Effect
0	Sorbitol (Lambros and Vanderberg, 1979)	Only ring stages remain
1	Magnetic separation (Ribaut et al., 2008)	Only late trophozoites remain
2	Heparin 250µg/ml (Boyle et al., 2010a)	No further invasion of erythrocytes
3	Magnetic separation	Only late trophozoites remain
4	Heparin	No further invasion of erythrocytes
7	Incubation with E64(5-8h)	No rupturing of schizonts
7	Filter through 1.2µm filter	Mechanical release of merozoites

The released merozoites were given in incomplete medium (without Albumax and serum), a suitable concentration of compounds and erythrocytes for 30 minutes for invasion of red blood cells. After that period, serum and Albumax was added.

Solithromycin treatment assay

In order to test the efficacy of solithromycin, the established IC50 and IC99 for *P. falciparum* blood stage parasites were tested (Wittlin et al., 2012).

Synchronised merozoites were equally divided into three T25 flasks and exposed to 3 nM, 10 nM solithromycin or 0.001% DMSO as control with a starting parasitaemia determined at 0.5% with a haematocrit of 1%. Development of the culture was observed over 96 hours by preparing thin blood smears.

Apicoplast free 3D7 drug assay

Depletion of the apicoplast of *P. falciparum* 3D7 was implemented by following the procedure by Yeh and DeRisi (Yeh and DeRisi, 2011). Briefly, parasites were treated with clindamycin in order to target the apicoplast. These treated parasites were subsequently supplemented with 200 µM IPP at several time points after the first cycle of multiplication in erythrocytes. This procedure was undertaken to rescue the parasite but dispose the prokaryotic organelle. Parasites were further cultured *in vitro* while changing the medium supplemented with IPP every two days. IPP concentration was reduced after adaption of the parasites in steps of 50 µM. Parasites used for the presented experiments were adapted to a concentration of 100 µM IPP.

Synchronised (see above) 3D7 and 3D7 apicoplast free (ap-) *falciparum* ring stages with a start parasitaemia of 0.5% and a haematocrit of 1% were subjected to the following experimental setup:

Table 2. Experimental setup of *P. falciparum* apicoplast free assay.

	Apicoplast free 3D7	**3D7**
Control	0.001 % DMSO + 100 µM IPP	0.001 % DMSO
Solithromycin	10 nM solithromycin + 100 µM IPP	10 nM solithromycin
Clindamycin	50 nM clindamycin + 100 µM IPP	50 nM clindamycin

The culture was observed over 120 h via thin blood smears.

2.3 Methods of *Plasmodium berghei* ANKA parasites

All experiments were undertaken with *P. berghei* ANKA cl15cy1 at the Parasitology Unit, Ann-Kristin Müller lab, Centre for Infectious Diseases, Heidelberg.

2.3.1 Imitation of the parasite's life cycle

For *Plasmodium berghei* and this experimental setup, it is necessary to imitate the life cycle of the parasite.

Eggs of female *Anopheles stephensi* mosquitos were collected 4 days after a blood meal (see below) and washed with 70% ethanol and 0.1% salt solution. They were bred in trays containing 0.1% salt solution at 28°C and 80% humidity. Two or three days after, the larvae were again split and fed with cat food. Within one week they developed into pupae. Two days later the hatched mosquitos were transferred into cages and fed via cotton pads soaked with 0.1% salt solution or 10% sucrose solution.

Malaria naïve female NMRI mice were infected with *P. berghei ANKA* positive mouse blood samples by *intraperitoneal* injection on two following days. Four days later, when exflagellation of male gametocytes in a thin blood smear could be observed, mice were anaesthetised with 70 - 100 µl Ketamine/Xylazine respectively. The mice were laid on a mosquito cage with 4 days old *Anopheles stephensi* for a 15 minutes blood meal for infection with *P. berghei ANKA.* Infected mosquitos were kept in an incubator at 21°C and 80% humidity.

Figure 11. *Anopheles stephensi* mosquito cages in incubator

Estimation of infection rate

After 10 days, mosquito infection was assessed by detecting the occurrence and number of oocysts in the midguts of *Anopheles* mosquitos.

Up to 5 mosquitoes were anaesthetised on ice and midguts were dissected in RPMI medium containing 3% BSA. Afterwards, the midguts were analysed by preparing them on a glass slide under a coverslip. Examination was performed by light microscopy using a 40x objective and phase contrast.

Hence, by observing the total number of infected midguts, infection intensity was estimated.

Isolation of sporozoites

Mosquitos were dissected 17 to 23 days after infection. To extract sporozoites out of the salivary gland, *Anopheles stephensi* mosquitos were anaesthetised on ice and dissected in RMPI supplemented 3% BSA under a stereomicroscope using insulin syringes and needles. Afterwards, salivary glands were homogenised using an EPPI-Pistill.

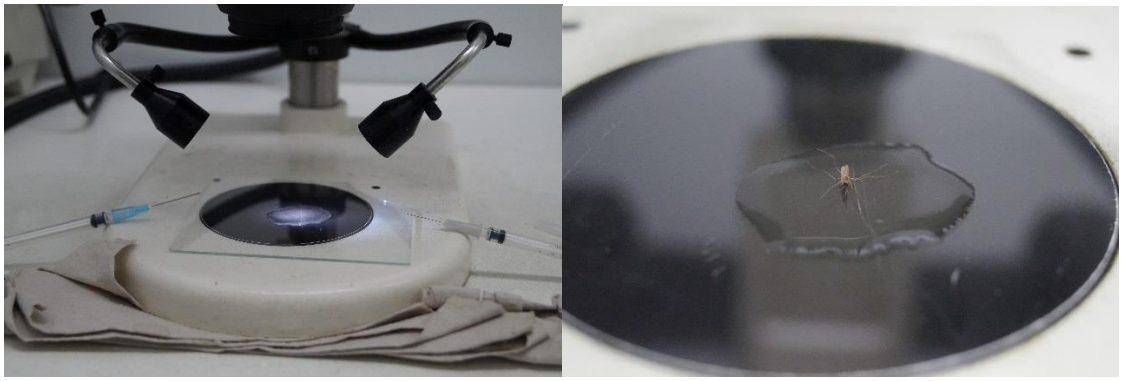


Figure 12. Dissection of *Anopheles stephensi* mosquitos

The homogenised salivary glands were centrifuged for 3 minutes / 1000 rpm at 4°C. The supernatant containing sporozoites was transferred into a new Eppendorf tube, the pellet resuspended in RPMI/ 3% BSA and again centrifuged. The new supernatant was transferred into the tube as well. To calculate the number of sporozoites, the suspension was diluted, placed into a hemocytometer and sporozoites were counted using light microscopy and phase contrast. The following formula was used to calculate the total number of extracted sporozoites:

$$average\ of\ \frac{sporozoites}{mm^2} \times\ dilution\ factor \times\ 10^4\ = \frac{sporozoites}{ml}$$

2.3.2 Culture conditions of HuH7 cell line and hNTCP Huh7 cell line

Culturing of hepatocytes was performed under sterile conditions.

The Huh7 required a culture medium containing DMEM Medium (supplemented with D-Glucose, L- Glutamine and Pyruvate), 10% FCS + 1% Anti – anti 100x.

The cell line hNTCP HuH7 was essential for the PreS1-conjugated drug assay. The PreS1 domain physiologically binds to the polypeptide hNTCP (see section 1.7) The hNTCP HuH7 cell line expresses this binding site, hence the PreS1 conjugated drug can be delivered into the hepatocyte. The cells were cultured in a medium containing DMEM Medium (also supplemented with D-glucose, L-glutamine and pyruvate), 10% FCS + 1% penicillin/streptomycin.

Both cultures were maintained in T25 or T75 culture flasks, in an incubator at 37°C and 5% O_2 and split regularly: cells were washed with prewarmed HBSS (without $CaCl_2$ and $MgCl_2$), incubated 3 minutes with prewarmed 0,25% trypsin-EDTA to solve them from the flask. Trypsinization was stopped by adding 10ml of cell culture medium and then centrifuged for 3 minutes / 1000 rpm. Up to 1 ml of the pellet was resuspended in culture medium and transferred into a new flask.

2.3.3 *In vitro* drug assay

Drug assays were performed under sterile conditions.

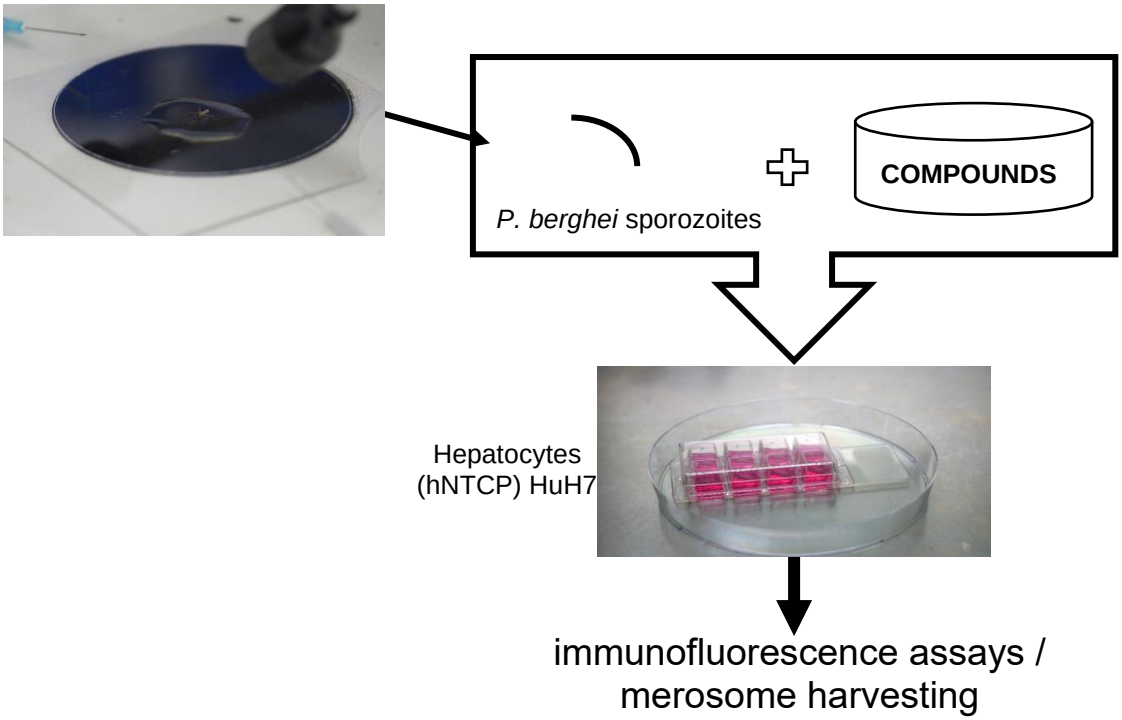


Figure 13. Experimental set-up: Effect of compounds on pre-erythrocytic development. After dissection, *P. berghei* sporozoites and compounds were added to (hNTCP) HuH7 and developed until stopped at different time points.

Infection of hepatocytes

One day prior to sporozoite infection, hepatocytes were seeded into 8-well chamber slides.

To achieve a total number of 25 000 cells/ well, cells were prepared as described above (see section 2.3.2). After centrifugation, an aliquot of the pellet was diluted 1:5 in Trypan blue, and counted by using a hemocytometer (formula as described above (see section 2.3.2). Afterwards, the cell suspension was diluted to achieve an overall number of 25 000 cells in 400 µl culture medium per well respectively. The chamber slides were incubated overnight at 37°C and 5% O_2. The following day sporozoites were extracted as described above (see section 2.3.1) and, depending on the experimental setup, 10 000 or 25 000 sporozoites, diluted in 100 µl RPMI, were pipetted in each well and incubated for 90 minutes at 37°C for sporozoite invasion of hepatocytes.

Drug treatment

After 90 minutes of incubation, 400 µL of supernatant containing sporozoites that failed to invade was removed from the wells. Subsequently, 400 µl of culture medium containing the needed concentration of compounds was replaced.

Solithromycin assay:

- solithromycin (solved in DMSO) 10 µM
- solithromycin (solved in DMSO) 1 µM
- azithromycin (solved in distilled H_2O) 1 µM
- control: 0.01% DMSO

Every compound was further diluted in HuH7 cell culture medium.

PreS1- conjugated drugs assay:

- primaquine (solved in PBS) 10 µM
- PreS1-primaquine (solved in DMSO) 10 µM
- control: 0.01 % DMSO

Every compound was further diluted in hNTCP HuH7 cell culture medium.

After incubation of 48 or 65 hours, slides were fixed for 10 minutes with ice-cold methanol, afterwards blocked with 10% FCS/PBS for 30 minutes at 37°C or overnight at 4°C.

Immunofluorescence staining

Antibodies were diluted in 10% FCS/PBS, washing steps were performed with 1% FCS/PBS.

To visualize the apicoplast of liver stage parasites, 100 µl of an anti-PbACP rabbit antiserum 1:1000 was applied and incubated for 1 hour at 37°C. The wells were then washed five times. Subsequently, 100 µl of a secondary antibody anti-rabbit Alexa Fluor 546 1:300 was applied and incubated for 45 minutes at 37°C.

After three washing steps, the wells were again blocked with 10% FCS/PBS for 15 minutes at 37°C.

After three times of washing, 100 µl of an anti- HSP70 mouse antibody 1:100 was applied and incubated for 30 minutes at 37°C. This was again followed by three times of washing and incubation with 100 µl of a secondary anti-mouse Alexa Fluor 488 antibody 1:300 for 20 minutes at 37°C.

Finally, DNA was stained by adding 100 µl Hoechst 33342 1:1000 to the previous antibody. Ten minutes later three washing steps followed. Slides were then mounted with 50% glycerol in PBS and coverslips fixed with nail polish.

Parasite numbers in each well were counted after 48 or 65 hours of development via epifluorescence microscopy. Sizes measured with the Fiji package in Image J.

Invasion assay

This assay was set up to test if solithromycin had an impact on the capability of *P. berghei* sporozoites to invade hepatocytes.

HuH7 cells were plated in chamber slides as described above (see section 2.3.2). In comparison to the treatment assay, the extracted sporozoites were incubated for 30 minutes in either 10 µM solithromycin/RPMI or 0.01% DMSO/RPMI (control) on ice. Afterwards 10 000 treated sporozoites were added to each well. After 90 minutes of invasion, slides were fixed with 4% PFA/PBS for 10 – 20 minutes at room temperature and blocked with 10% FCS/PBS for 30 minutes at room temperature or overnight at 4°C.

Subsequently, immunofluorescence staining was performed to show the ratio of the number of sporozoites that successfully invaded vs. the number of sporozoites that failed to invade.

To visualize the sporozoites which were not able to invade hepatocytes, an anti-PbCSP mouse antibody 1:300 was added to each well and incubated for 1 hour at 37°C. After four times of washing, a secondary goat anti-mouse Alexa Fluor 546 antibody 1:300 was added to each well and incubated for 1 hour at 37°C as well and afterwards washed four times. A fixing step with ice-cold methanol for 10 minutes at room temperature followed by 30 minutes of blocking with 10% FCS/PBS was performed. As a result of this treatment, the antibodies can penetrate into the cells and stain intra-hepatocytic sporozoites. Consequently, to stain all sporozoites, the anti-PbCSP mouse antibody 1:300 was again added to each well but this time, a goat anti-mouse Alexa Fluor 488 1:300 followed, and was also incubated for 1 hour at 37°C.

The slides were mounted and covered as described above (see section 2.3.3). The number of sporozoites was counted via immunofluorescence microscopy. The following formula for the calculation of the proportion of successfully invaded sporozoites was used:

$$\frac{(total\ \ number\ of\ sporozoites - not\ invaded\ sporozoites)}{total\ number\ of\ sporozoites}\ x\ 100$$

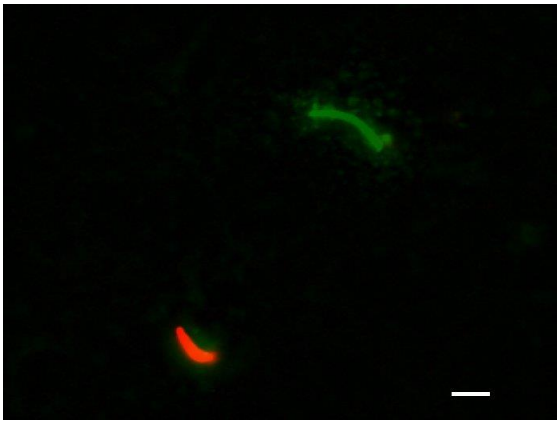


Figure 14. Invaded and failed to invaded *P. berghei* sporozoite. Due to the special order of staining processes, sporozoites which did not invade hepatocytes appear red while all sporozoites (invaded and not invaded) appear green. Scale bar indicates 5µm.

2.4 *In vivo* experiments with *P.berghei* ANKA

All *in vivo* experiments were approved by the relevant authority (Regierungspräsidium Karlsruhe no. G260/12), performed under European regulations and supervision by an appropriated person.

NMRI mice were kept in the animal shelter of the University of Heidelberg.

2.4.1 Culturing of *P. berghei* liver stage merosomes

Merosome formation requires an incubation time of 70-75 hours of *P. berghei* liver stages. Therefore 8 well- chamber slides were seeded with HuH7 cells, each well was infected with 50 000 sporozoites and treated with either 10 µM solithromycin or 0.01% DMSO as described above (see section 2.3.3.). The slides were incubated for 72 hours at 37°C and cell culture medium containing compound or control was changed daily.

72 hours after infection, merosomes appeared in the supernatant of the wells. Hence, the supernatant was carefully pipetted up and down and transferred in an Eppendorf tube, which was centrifuged for 5 minutes / 1200 rpm at room temperature.

After centrifugation, the supernatant was taken off and 400 µl of culture medium was applied to wash out the drug. The tubes were centrifuged again and the supernatant was taken off. Afterwards, the merosome containing pellets were suspended and merosomes examined and counted using light microscopy and phase contrast with a hemocytometer. The total number of merosomes was calculated and diluted for injection.

2.4.2 Infection of NMRI mice with *P. berghei* liver stage merosomes

A suitable amount of merosomes was injected i.v. into the tail vein of NMRI mice using an insulin syringe (U100, 12,7 mm, 30G). After 3 days of infection, thin blood smears were performed daily to detect parasitaemia.

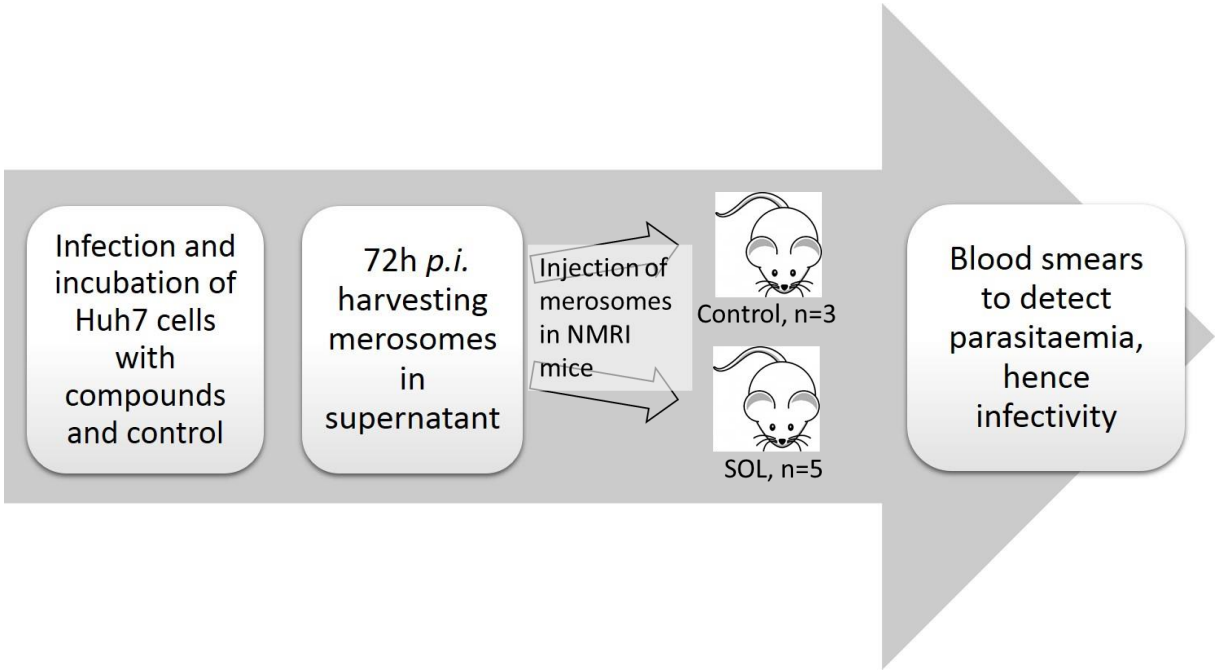


Figure 15. Experimental setup: effect of solithromycin on merosome infectivity. Hepatocytes were infected with *P. berghei* sporozoites and simultaneously treated with solithromycin. Merosomes were harvested 72 hours after infection and injected into NMRI mice. Parasitaemia was controlled via thin blood smear.

2.5 Statistics

Graph Pad Prism was used for statistical analyses.

Gaussian distribution was estimated applying D'Agostino and Pearson omnibus normality test plus Kolmogorov-Smirnov test.

P values were calculated using the student's t-test if Gaussian distribution was confirmed or Mann-Whitney test if not. Data that are normally distributed were summarized using descriptive statistics, e.g., using the mean (SD). Non-parametric data were summarized using the median and the interquartile range or range.

3 Results

3.1 Effect of solithromycin on *P. falciparum* 3D7 blood stage parasites

Initially, I was keen to verify the effect of solithromycin on *P. falciparum* blood stage parasites. Special focus was on whether the compound uses the apicoplast as a target or not. Since it is known that inhibiting the apicoplast's inheritance results in delayed death of blood stage parasites, I focused especially on the parasites' development over 96 hours, hence more than one multiplication cycle.

I tested the effect of two published concentrations (IC_{50} of 3 nM and IC_{99} of 10 nM) on *P. falciparum* 3D7 blood stage proliferation (Wittlin et al., 2012). I applied 1 µM of azithromycin as a reference for the 'delayed death' phenotype induced by interfering with prokaryotic protein translation in the apicoplast (Friesen et al., 2010).

3.1.1 Effect of solithromycin on erythrocyte invasion of *P. falciparum* 3D7 blood stage merozoites

To investigate whether solithromycin has an influence on merozoite invasion of erythrocytes it is necessary to test the compound at time point zero after merozoites emerge from mature schizonts but before invasion of erythrocytes. Therefore, *P. falciparum* merozoites were isolated as described above (see section 2.2.2) and exposed to the compound during invasion, i.e., when freshly isolated merozoites were added to uninfected erythrocytes suspended in culture medium.

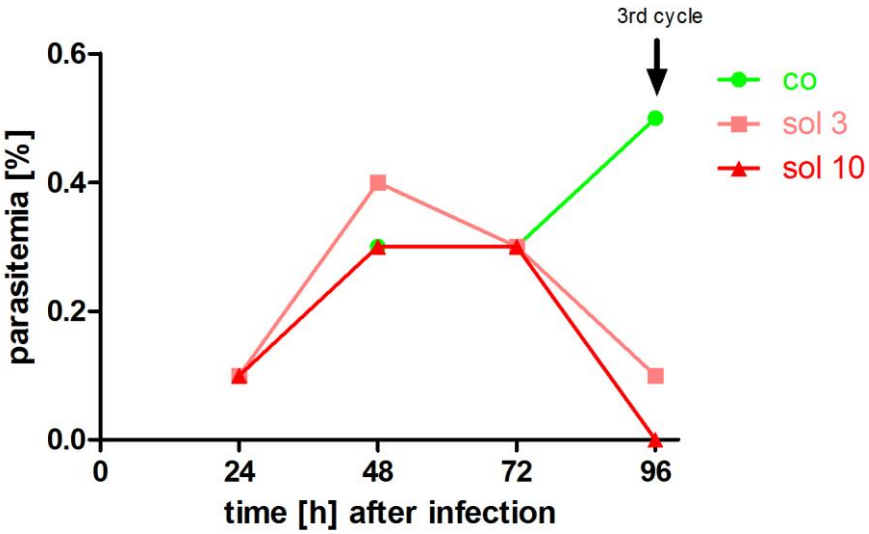


Figure 16. Exposure to solithromycin did not affect the initial merozoite invasion of erythrocytes but shows delayed death of parasites. *P. falciparum* merozoites were exposed to different concentrations of solithromycin (sol 3 = 3 nM solithromycin; sol 10 = 10 nM solithromycin; co= 0.001% DMSO) and observed over 96h.

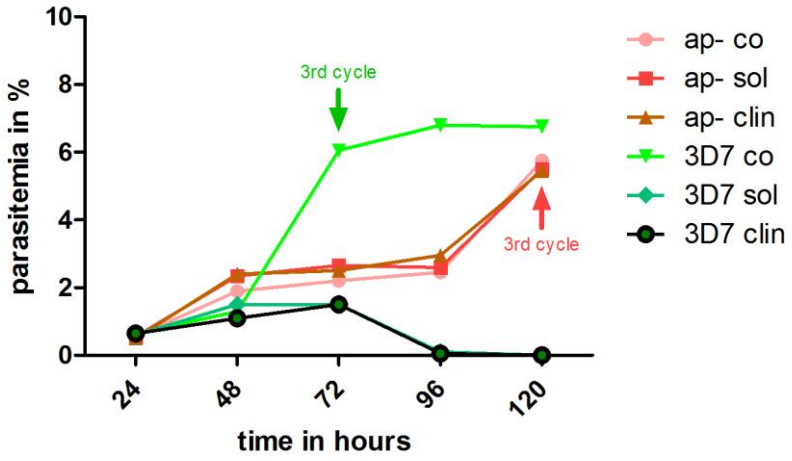
Figure 16 depicts the growth of *P. falciparum* blood stage parasites after exposure of merozoites to solithromycin; a concentration of 3 nM corresponds to the IC_{50}, 10 nM the IC_{99}. The compound did not inhibit merozoite invasion of erythrocytes. Solithromycin exposed cultures grew at the same rate as the untreated control culture over the first two cycles of exposure. However, I observed the delayed death of solithromycin exposed cultures after 96 hours of infection, which suggested a defect of apicoplast inheritance.

3.1.2 Effect of solithromycin on *P. falciparum* blood stage parasites lacking the apicoplast

To test this hypothesis, I tested the compound on *P. falciparum* blood stages lacking the apicoplast. Parasites were treated with clindamycin and supplemented with IPP to achieve removal of the apicoplast while keeping the parasite alive. IPP is the only essential metabolic product of the apicoplast in *Plasmodium* blood stages (see section 1.3.1). Apicoplast-negative parasites were designated as ap-. Synchronised *P. falciparum* 3D7 and *P. falciparum* 3D7 ap- ring stages were treated as described above (see section 2.2.2) with either

10 µM solithromycin, 50 nM clindamycin as positive or 0.001% DMSO as negative control. Parasitaemia was monitored via thin blood smears over 120 hours.

A


parasitemia in %
10
8
6
4
2
0
3rd cycle
3rd cycle
24
48
72
96
120
time in hours
ap- co
ap- sol
ap- clin
3D7 co
3D7 sol
3D7 clin

B

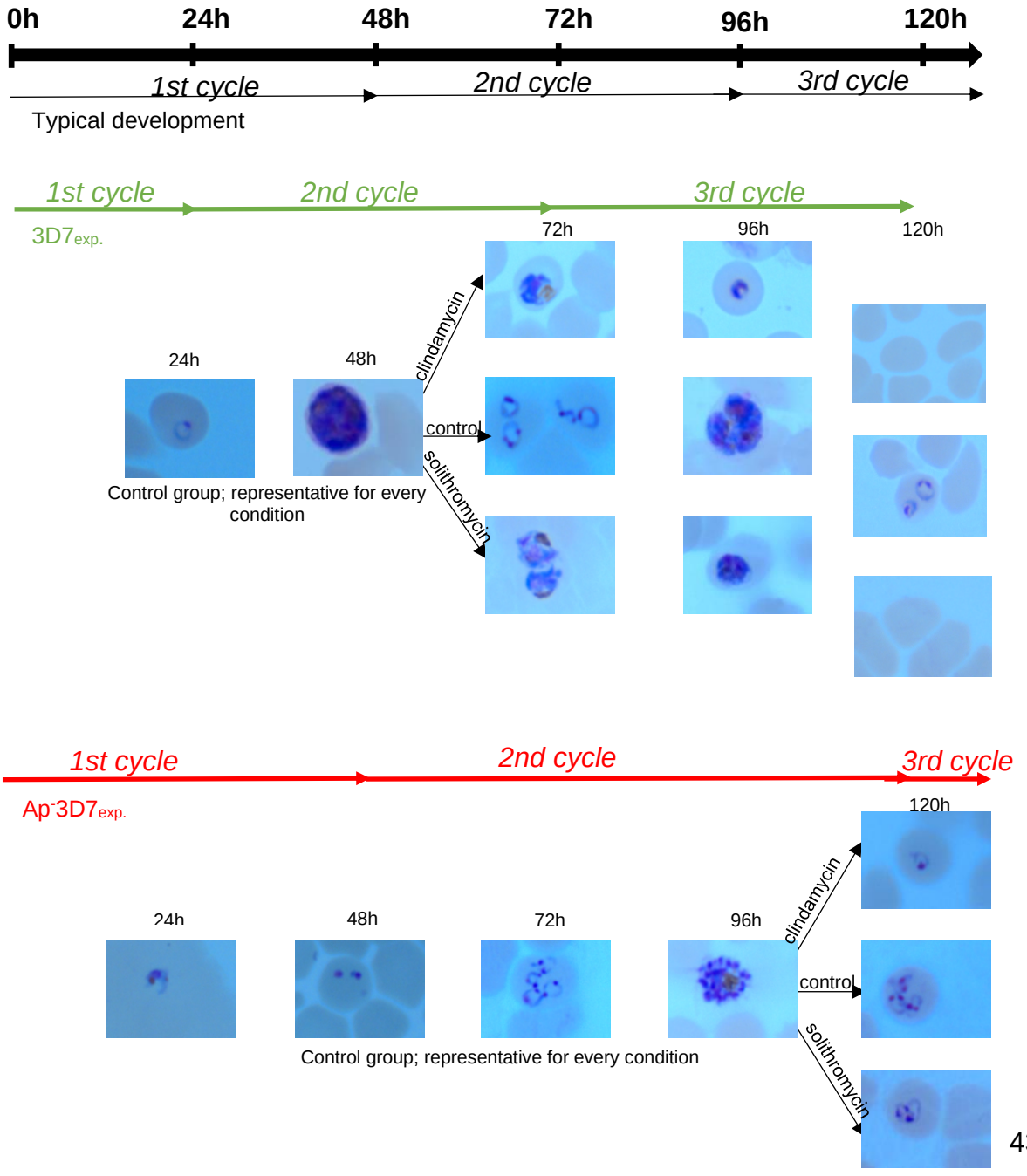

0h
24h
48h
72h
96h
120h
1st cycle
2nd cycle
3rd cycle
Typical development
1st cycle
2nd cycle
3rd cycle
3D7exp.
72h
96h
120h
24h
48h
clindamycin
control
solithromycin
Control group; representative for every condition
1st cycle
2nd cycle
3rd cycle
Ap-3D7exp.
120h
24h
48h
72h
96h
clindamycin
control
solithromycin
Control group; representative for every condition

Figure 17. Growth of apicoplast free *P. falciparum* blood stage parasites was not affected by solithromycin treatment. Synchronised ring stages were incubated with compounds and observed over 120h. A) Control 3D7 parasites turned into the third cycle of development after 72h while treated parasites stayed in the second cycle. (= delayed death). Apicoplast free 3D7 parasites supplemented with IPP got into their third cycle after 120h, even parasites under drug treatment. B) Microscopy pictures of developed parasites. Solithromycin and clindamycin treated *P. falciparum* 3D7 with apicoplast did not develop further than two cycles of schizogony, stayed in schizont stage and died eventually. Whereas the parasites lacking the apicoplast but supplemented with 100 µM IPP continued their development. Means of two independent experiments.

As shown in Figure 17, ap- parasite growth was not affected by treatment with solithromycin or clindamycin. Solithromycin and clindamycin treated parasites from the lab strain 3D7 showed the typical delayed death: treated parasites were not able to develop further than a second cycle of erythrocyte invasion.

Compared to the wildtype *P. falciparum* lab strain 3D7 ap- parasites were slightly delayed in their development.

3.2 Effect of solithromycin on *P. berghei* Huh7 liver stage parasites

After receiving the results of solithromycin's effect in blood stage parasites, suspecting that the compound targets the apicoplast, I continued my experiments focusing on the liver stage of *Plasmodium* parasites.

To find out if solithromycin could be a candidate drug for a chemo attenuated whole parasite vaccine protocol, its potency and detailed mode of action against liver stages needs to be demonstrated. To start this analysis, I held on to the life cycle of the parasite.

I started this analysis by treating *P. berghei* ANKA sporozoites with the compound to detect a possible effect (see section 2.3.3). As a second step, I infected HuH7 hepatocytes with *P. berghei* ANKA sporozoites as described in material and methods (see section 2.3.3). Developing liver stage parasites were treated with either 1 µM or 10 µM solithromycin, the corresponding concentration

of DMSO served as control. I used 1µM azithromycin as a positive control due to its known effect described above (see section 1.3.2).

3.2.1 Effect of solithromycin on invasion rates of *P. berghei* sporozoites in hepatocytes

To be a usable candidate, solithromycin should not inhibit the invasion capability of sporozoites. Initially, I tested the potential inhibitory potency of solithromycin on hepatocyte invasion. *P. berghei* sporozoites were incubated with either 10 µM solithromycin or 0.01% DMSO for 1 hour prior to invasion of HuH7 hepatocytes. Sporozoites, which were unable to invade hepatocytes were stained with an anti-PbCSP antibody and Alexa fluor 546 as a secondary antibody, before visualisation via fluorescence microscopy. Afterwards, all sporozoites regardless of invasion status were stained by the anti-PbCSP antibody and Alexa fluor 488 as secondary antibody, again before fluorescence microscopy.

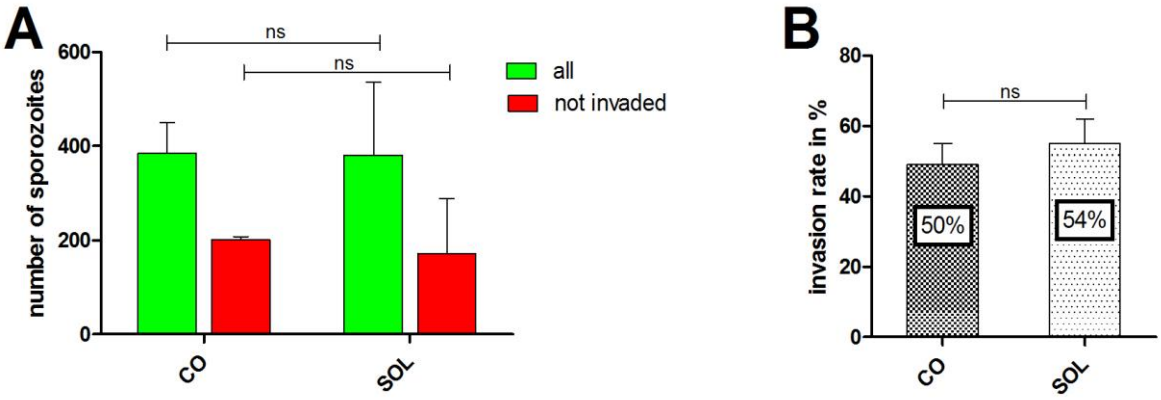


Figure 18. Solithromycin did not affect the capacity of *P. berghei* sporozoites to invade hepatocytes. Sporozoites were incubated with 10 µM solithromycin or 0.01% DMSO as control and afterwards invaded hepatocytes for 90 minutes. A) There was no significant difference in the number of sporozoites that successfully invaded or not between control and solithromycin. B) The invasion rate of treated and untreated sporozoites showed no significant difference. Data from three independent experiments. ns= non-significant, Mann-Whitney U test. Error bars indicate median and range.

Figure 18 illustrates the overall number of sporozoites that did or did not successfully invade after 90 minutes. As shown, the incubation with solithromycin prior to hepatocyte invasion did not affect the number of successfully invaded parasites.

Following this observation, I used an extended experimental setup to investigate whether intrahepatocytic parasite numbers are affected by solithromycin treatment at 65 hours post invasion.

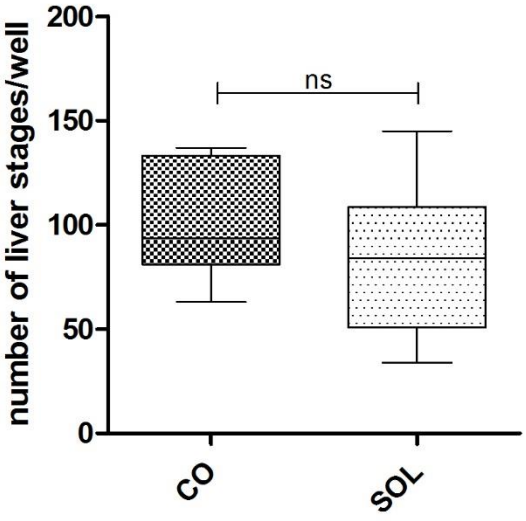


Figure 19. Incubation of sporozoites with solithromycin prior to invasion did not block the development of liver stage parasites after 65 hours. Treated sporozoites were counted after 65h of development. CO = 0.01% DMSO (n= 6); SOL = 10 µM solithromycin (n= 10). Data represent two independent experiments. ns= non-significant, Mann-Whitney U test. Whiskers indicate median and range.

As shown in Figure 19, the number of *P. berghei* liver stages, which developed from with sporozoites that were pre-incubated with solithromycin, was not significantly different from the control group.

3.2.2. Analysis of parasite development in HuH7 hepatocytes

P. berghei liver stage parasites were cultured and treated with the given compounds. Cultures were stopped at 48h or 65h of incubation. Slides were fixed, stained and counted via epifluorescence, as described above (see section 2.3.3)

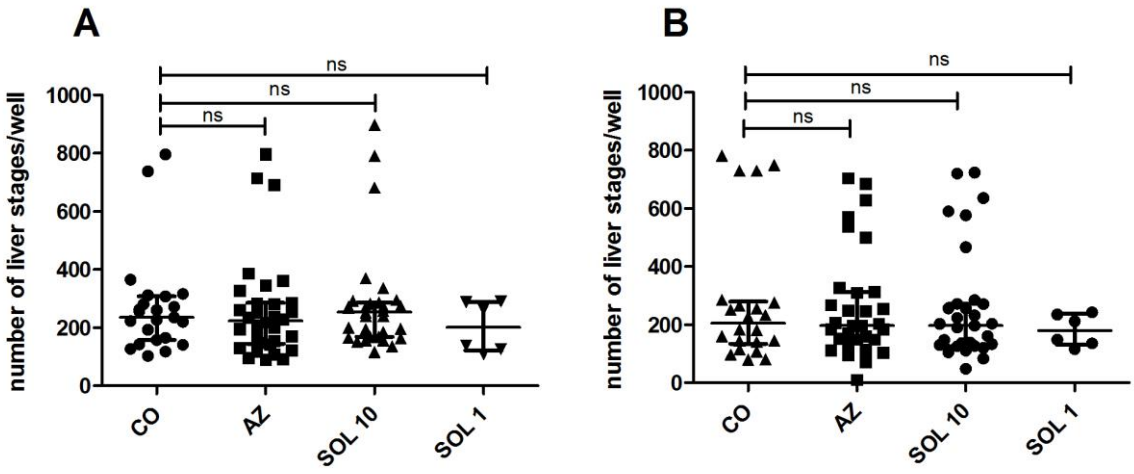


Figure 20. Treatment of *P. berghei* liver stages did not affect the number of liver stage parasites. Dot plot showing the number of mature liver stages under different antibiotic treatments. Every point represents one well on an 8-well chamber slide. A) Number of liver stages 48h after infection CO= 0.01% or 1% DMSO (n = 23); AZ= 1 µM azithromycin (n =32); SOL10 = 10 µM solithromycin (n= 32) SOL 1= 1µM solithromycin (n= 6) B) number of parasites 65h after infection. CO= 0.01% or 1% DMSO (n= 22); AZ= 1 µM azithromycin (n= 31); SOL10= 10 µM solithromycin (n= 31) SOL 1 = 1 µM solithromycin (n= 6). ns= non-significant, Mann-Whitney U test. Whiskers indicate median and interquartile range.

Figure 20 reveals that none of the antibiotic treatments led to changes in the number of liver stages, neither after 48 nor after 65 hours. Outliers are due to inter-experimental variation (batch effect), which is shown in Figure 21: same colours represent a chamber slide, hence one experiment.

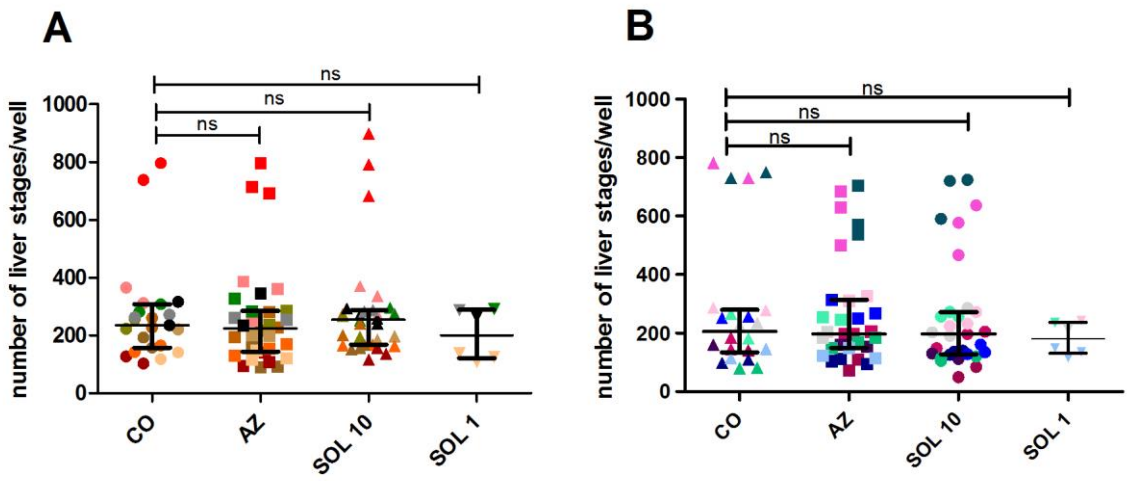


Figure 21. Analysis of outliers in the comparison of the number of *P. berghei* liver stages under antibiotic treatment. Dot plot with each colour representing one experiment, every data point representing a single well on a chamber slide. A) number of parasites 48h after infection CO= 0.01% or 1% DMSO (n = 32); AZ= 1 µM azithromycin (n =32); SOL10 = 10 µM solithromycin (n= 32); SOL 1= 1µM solithromycin (n= 6) B) Number of parasites 65h after infection. CO= 0.01% or 1% DMSO (n= 22); AZ= 1µM azithromycin, (n= 31); SOL10= 10µM solithromycin (n= 31); SOL 1= 1µM solithromycin (n= 6). ns= non-significant, Mann-Whitney U test. Whiskers indicate median and interquartile range.

In summary, solithromycin did not affect the number of liver stage parasites within the HuH7 hepatocytes.

3.2.3 Effect of solithromycin on the maturation of *P. berghei* liver stage parasites

As described in material and methods (see section 2.3.3), *P. berghei* liver stages were cultured and their sizes measured after 48h and 65h of development within hepatocytes.

In the beginning, experiments were undertaken with a concentration of 1% DMSO as control and 10 µM solithromycin solved in DMSO (resulting in a final concentration of 1% DMSO in solithromycin wells)

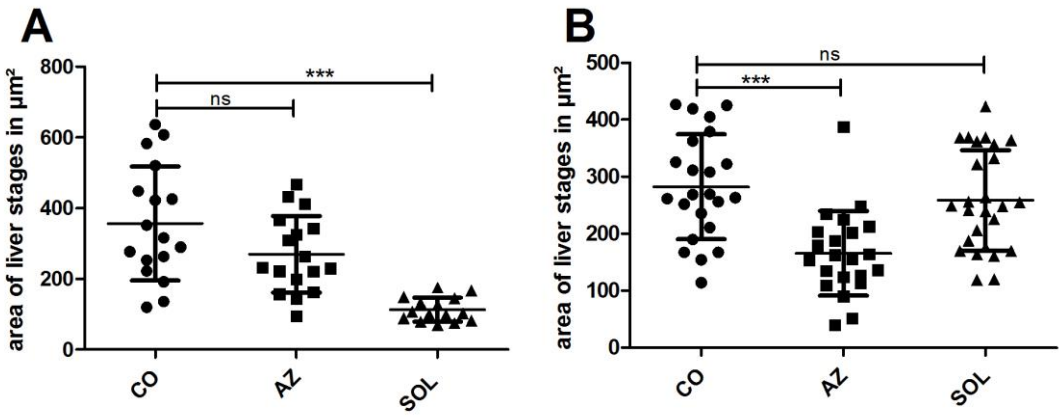


Figure 22. Calculated areas of *P. berghei* liver stages under antibiotic treatment with 1 % DMSO as a control. Dot plot comparing calculated areas of liver stage parasites under different antibiotic treatments. Every point represents one liver stage parasite. A) Sizes of parasites after 48h. CO= 1% DMSO (n= 17); AZ= 1 µM azithromycin (n= 17), SOL = 10 µM solithromycin (n= 15). B) Sizes of parasites after 65h. CO= 1 % DMSO (n= 23); AZ= 1 µM azithromycin (n= 22); SOL = 10 µM solithromycin (n= 26). ns= non-significant, *** p< 0.0001, student's t-test. Whiskers indicate mean and SD.

Demonstrated in Figure 22, treatment with azithromycin did not affect the sizes of developed parasites after 48h of development but after 65h. However, there was a significant difference in sizes of solithromycin treated parasites in the earlier time point, but not after 65 hours of development.

It is known that DMSO not only has a cytotoxic effect but can also intensify the cytotoxicity of drugs. Additionally, it has a modulating effect on HuH7 cells (Liu et al., 2015). Therefore, I changed the concentration of DMSO concentration in the solithromycin treated group and consequently, the control group, for subsequent experiments to 0.01%. Furthermore, I also lowered the concentration of solithromycin to see if the concentration of the compound plays a role in this observation.

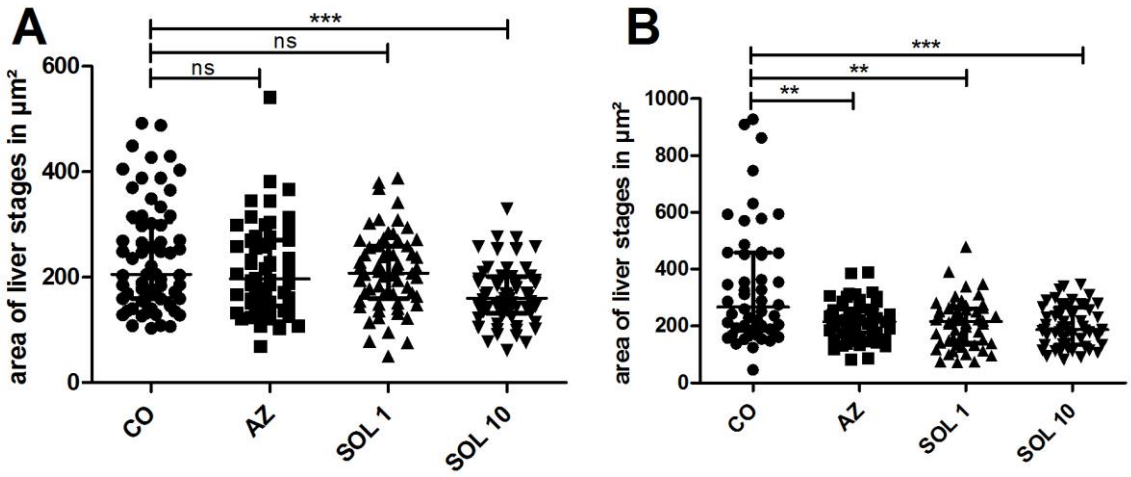


Figure 23: Sizes of *P. berghei* liver stages under antibiotic treatment with 0.01 % DMSO as control. Dot plot comparing measured sizes of liver stage parasites under different antibiotic treatments. Every dot represents one liver stage parasite. A) Sizes of parasites after 48h CO= 0.01% DMSO, (n= 66); AZ= 1 µM azithromycin (n= 60); SOL 1= 1 µM solithromycin (n= 61); SOL 10= 10 µM solithromycin (n= 62) B) sizes of parasites after 65h. CO= 0.01 % DMSO (n= 50); AZ= 1 µM azithromycin (n= 55); SOL 1= 1 µM solithromycin (n= 54); SOL 10= 10 µM solithromycin (n= 56). ns= non-significant. ***p<0.0001, **p<0.01, Mann-Whitney test. Median with interquartile range given.

Figure 23 shows the sizes of liver stages at the two time points. Again, my results from the earlier timepoint show that parasites treated with 10 µM solithromycin were significantly smaller compared to the control group. However, after 65 hours of development, every antibiotic treatment causes a significant difference in the sizes of measured parasites. Furthermore, the distribution of sizes is affected by antibiotic treatment after 65 hours of development with absence of large parasites. Treatment with 10 µM solithromycin affects parasites' sizes after 48 and 65 hours of development. Whereas the lower concentration of 1 µM solithromycin, as well as the treatment with 1 µM azithromycin, merely shows reduced sizes of liver stages after 65 hours of exposure.

3.2.4 Effect of DMSO concentration on *P. berghei* liver stage parasites

Since decreasing the concentration of DMSO from 1% to 0.01% resulted in different outcomes, I was interested in comparing the same antibiotic treatment conditions with respect to the DMSO concentration.

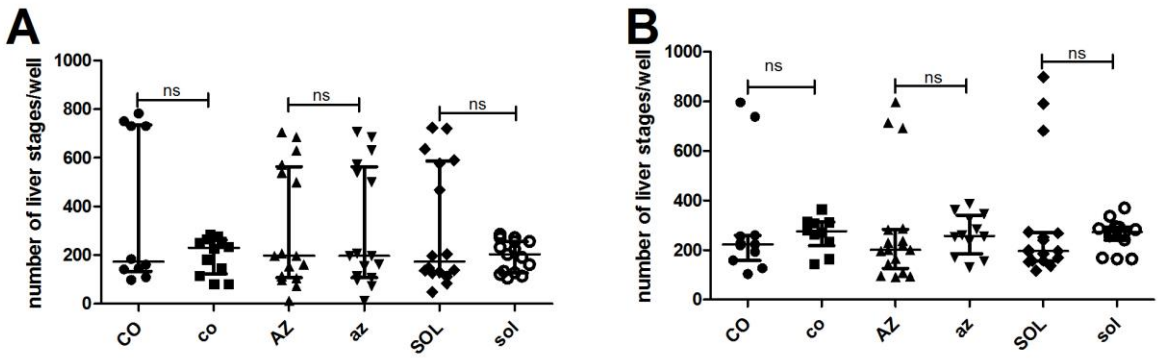


Figure 24. Different DMSO concentrations did not influence the number of liver stage parasites. Dot plot comparing liver load under different experimental conditions. Capital letters= experiments with 1% DMSO as control and solithromycin solvent, small letters= experiments with 0.01% DMSO. every dot represents a liver stage parasite A) number of parasites after 48h of development CO= 1% DMSO (n=11); co= 0.01% DMSO (n=10); AZ= 1 µM azithromycin while control 1% DMSO (n= 17); az= 1µM azithromycin while control 0.01% DMSO (n=12); SOL= 10 µM solithromycin and 1% DMSO concentration (n=17) sol= 10µM solithromycin and 0.01% DMSO (n=15) B) number of parasites after 65h of development. CO= 1% DMSO (n=10); co= 0.01% DMSO (n=12); AZ = 1 µM azithromycin while control 1% DMSO (n= 16); az= 1µM azithromycin while control 0.01% DMSO (n=16); SOL= 10 µM solithromycin and 1% DMSO concentration (n=16) sol= 10µM solithromycin and 0.01% DMSO (n=15). ns= non-significant, Mann-Whitney test. Whiskers indicate median and interquartile range.

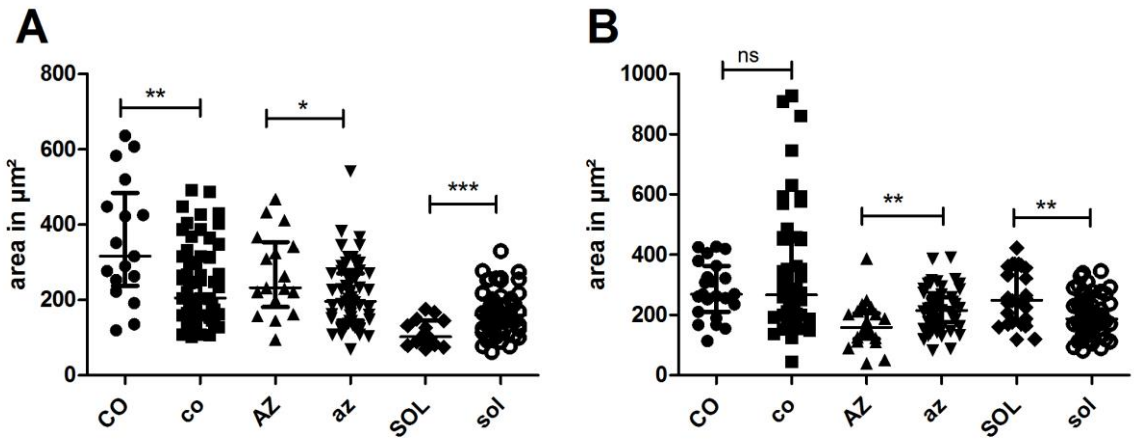


Figure 25. DMSO concentration had an influence on sizes of liver stage parasites. Dot plot comparing sizes of liver stage parasites under different experimental conditions. Capital letters= experiments with 1% DMSO, small letters= experiments with 0.01% DMSO, every dot represents a liver stage parasite A) Sizes of parasites after 48h of development. CO= 1% DMSO (n=17); co= 0.01% DMSO (n=66); AZ= 1 µM azithromycin while control 1% DMSO (n= 17); az= 1µM azithromycin while control 0.01% DMSO (n=60); SOL= 10 µM solithromycin and 1% DMSO concentration (n=15) sol= 10µM solithromycin and 0.01% DMSO (n=62) B) sizes of parasites after 65h of development. CO= 1% DMSO (n=23); co= 0.01% DMSO (n=50); AZ= 1 µM azithromycin while control 1% DMSO (n= 22); az= 1 µM azithromycin while control 0.01% DMSO (n=55); SOL= 10 µM solithromycin and 1% DMSO concentration (n=26) sol= 10 µM solithromycin and 0.01% DMSO (n=56). ns= non-significant, *p<0.1, **p<0.01, ***p<0.001, Mann Whitney and student's t-test. Whiskers indicate median and interquartile range.

As Figure 24 and 25 demonstrate, different DMSO concentrations did not influence parasite load in the liver but the individual sizes of the parasites while outliers again could be due to experimental repetition. Liver stage parasites in the control group with 0.01% DMSO were significantly smaller after 48h of development, but there was no difference at the later timepoint.

The azithromycin treatment also showed a significant difference: after 48h parasites were smaller in the 0.01% DMSO concentration setup, however after 65h of development they were significantly bigger in the 0.01% DMSO solution.

In contrast, solithromycin treatment in 0.01% DMSO resulted in larger parasites after 48h than in 1% DMSO. However, at 65h this difference was turned upside down, as parasites exposed to solithromycin in 0.01% DMSO were smaller in comparison to parasites exposed to the same concentration of solithromycin in 1% DMSO.

From this data, I conclude that DMSO concentrations had an inconsistent impact on the sizes of liver stage parasites.

3.2.5 Morphological changes of the apicoplast induced by solithromycin exposure

While its inheritance, the apicoplast of a *Plasmodium* parasite assumes a branching structure in order to distribute to every daughter cell 1:1. Therefore, *P. berghei* liver stages were stained 65 hours after infection as described above (see section 2.3.3) to visualise the apicoplast.

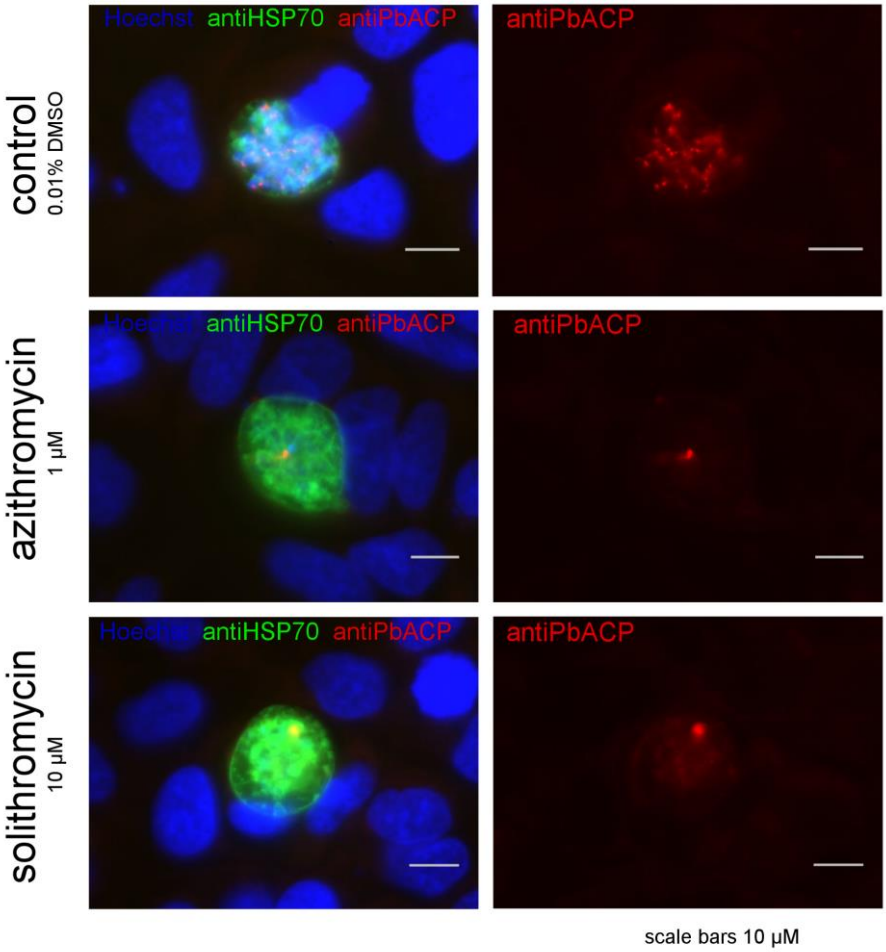


Figure 26. Visualisation of *P. berghei* liver stages 65 hours after hepatocyte infection shows a defect in the biogenesis of the apicoplast within solithromycin treated parasites. Parasites were stained with Hoechst for DNA staining, anti-HSP70 antibody to visualise the parasite itself (cytoplasm) and anti-PbACP serum to stain the apicoplast within the parasite. Treatment with azithromycin and solithromycin caused an arrest of apicoplast biogenesis in comparison to the control that shows a normal branching structure at this time point of development. Scale bars 10 µM.

Visualised in Figure 26, treatment with the bacteriostatic antibiotics azithromycin and solithromycin appears to block apicoplast biogenesis within the parasite after 65h of development. In the control group, the organelle displays the typical branching structure whereas antibiotic treated parasites remain with a punctuated apicoplast. This leads to the speculation that the tested antibiotics inhibit the inheritance of the prokaryotic organelle within the parasite: the apicoplast might not be distributed to every merozoite developing in a liver schizont

3.3 Infectivity of merosomes emerging from solithromycin treated *Plasmodium berghei* liver stage parasites

My results of the *in vitro* experiments identified the apicoplast as the target organellar structure of solithromycin.

To continue, the effect of solithromycin on liver stage merosomes and their infectivity *in vivo* was investigated. NMRI mice were infected with solithromycin treated merosomes, which emerged from infected HuH7 hepatocytes.

3.3.1 Identifying the best time point of *P. berghei* merosome harvesting and occurred numbers of merosomes

P. berghei liver stage parasites were cultured as described above (see section 2.3.3). However, the *in vivo* experiment required a higher amount of sporozoites per well (see section 2.4.1) to harvest a suitable amount of emerging merosomes from the supernatant collected from individual wells.

Table 3. Number of merosomes in two 8-well chamber slides cultured in 1% DMSO. Solely after 72 h of development, a single merosome was detected in the supernatant of a total of 16 wells.

Timepoint	Number of merosomes
65 h	0
70 h	0
72 h	1

The first attempt for selecting the correct timepoint for harvesting the maximum amount of merosomes was undertaken with DMEM medium containing 1% DMSO. However, there was no merosome in the supernatant of two 8-well chamber slides after 65 h or 70 h of parasite development. I found one single merosome emerging from a total of 16 wells after 72 hours.

To see whether the DMSO concentration had an impact on this observation, I repeated the experiment with an 8-well chamber slide of which four wells contained pure DMEM medium as control.

Table 4. Number of occurred merosomes in one 8-well chamber slide cultured in either DMEM supplemented with 1% DMSO or DMSO-free DMEM. In pure DMEM medium, merosomes emerged both earlier and in a higher number than in the four wells containing DMEM supplemented with 1% DMSO.

Timepoint	Number of merosomes in 4 wells containing 1% DMSO	Number of merosomes in 4 wells containing pure DMEM
70 h	0	139
72 h	8	+ 15

The data presented in Tables 3 and 4 show that 1% DMSO had a great impact on the numbers of merosomes and the time point of when they are released into the supernatant. Consequently, I decreased the concentration of DMSO for the control group as well as for the different treatments to 0.01%.

3.3.2 Morphology and number of merosomes emerging from *P. berghei* liver stage cultures exposed to solithromycin

While harvesting the emerged merosomes from the supernatant I observed that exposure of cultured *P. berghei* liver stages to solithromycin had an impact on the morphology and quantity of emerging merosomes.

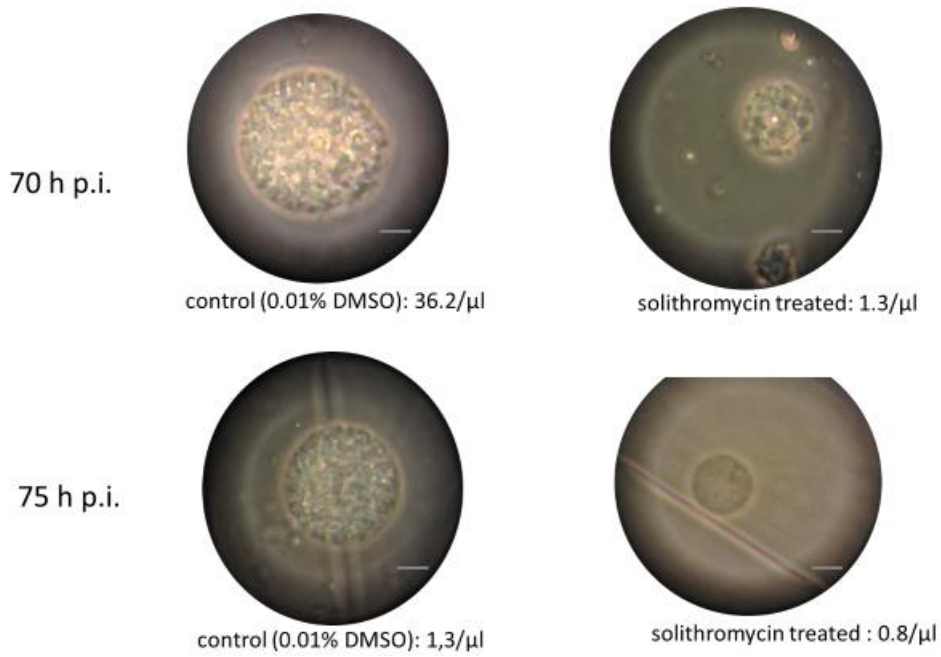


Figure 27. Treatment with solithromycin alters merosome formation (morphology and quantity). Comparison of measured concentration and appearance of merosomes emerging from treated and non-treated liver stage parasites at two different time points. Scale bars 10 µm.

As Figure 27 illustrates, 70 hours post hepatocyte infection, merosomes, which emerged from liver stages cultured in the presence of solithromycin, were smaller and their number in the supernatant was lower compared to control cultures. Additionally, the membranes of merosomes from solithromycin exposed cultures appeared to be less well defined and the merozoites themselves seemed to be condensed. 75 hours after hepatocyte infection, merosomes from solithromycin exposed cultures appeared empty. I observed the same phenotype also for different, including earlier, timepoints (65h p.i., 72h p.i., 77h p.i., data not shown).

3.3.3 Infectiousness of *P. berghei* merosomes from solithromycin-exposed liver stage cultures in NMRI mice

Next, I asked myself whether merosomes harvested from solithromycin exposed liver stages are infectious. To address this question, I injected a fixed amount of merosomes into the tail vein of malaria naïve female NMRI mice. By using a very thin insulin syringe (30G), I ensured that merosomes would be mechanically ruptured and therefore, merozoites could be released. Merosomes were

harvested from liver stages cultured under control conditions (0.01% DMSO) or cultured in 10 µM of solithromycin. Parasitaemia of mice was monitored daily via microscopic assessment of Giemsa stained thin blood smears.

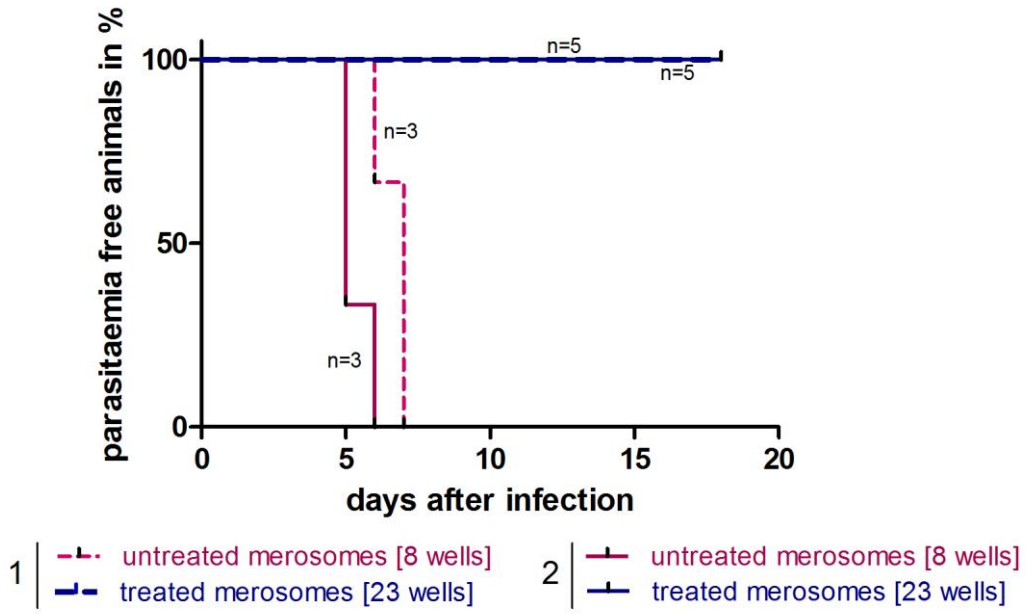


Figure 28. Kaplan-Meier analysis of time to blood stage patency in NMRI mice challenged with merosomes harvested from solithromycin exposed liver stage cultures. NMRI mice were infected with merosomes harvested from liver stage cultures exposed to either 10 µM solithromycin (all merosomes harvested from 23 wells were injected) or 0.01% DMSO (all merosomes harvested from 8 wells were injected). Parasitaemia was monitored via daily thin blood smears. 1) Mice infected with untreated merosomes (n= 5, 410 merosomes respectively) became patent after 6 or 7 days whereas mice infected with merosomes from solithromycin treated liver stage cultures (n=3, 210 merosomes respectively) did not develop parasitaemia. 2) Repetition experiment. Mice infected with untreated merosomes (n= 5, 440 merosomes respectively) became patent after 5 or 6 days whereas mice infected with merosomes from solithromycin exposed liver stage cultures (n=3, 424 merosomes respectively) remained free of parasitaemia.Figure 28 shows the parasitaemia progress of NMRI mice infected with either treated or control liver stage merosomes. It is shown that injection of merosomes, emerging from with solithromycin treated liver stage parasites, is not followed by blood stage infection. Hence, these merozoites are

in contrast to naïve merosomes not able to infect erythrocytes and therefore do not cause malaria symptoms.

Solithromycin inhibits the capacity of F1 progeny *P. berghei* merozoites emerging from exposed liver stage cultures to infect erythrocytes.

3.4 Effect of primaquine conjugated to hepatitis B Virus surface derived PreS-peptide on *P. berghei* liver stages cultured in transgenic human hepatoma cells that constitutively express NTCP

A potential strategy to gain liver specific transportation is based on the discovery of the peptide domain (PreS1) of the hepatitis B virus surface antigen that mediates highly specific binding to the sodium taurocholate co-transporting polypeptide (NTCP) on the surface of hepatocytes (see section 1.7).

This liver specific transportation is interesting for the use in whole organism vaccinations. The conjugated drugs could be applied simultaneously with the parasites and be delivered specifically to the liver. This could result in less systemic adverse side effects, dose reduction of the drug and improvement of patients' compliance.

To study the effectiveness of a PreS-conjugated drug regarding the usage in whole organism vaccination, I used a well-studied and known anti-malarial which is called primaquine. Besides the interesting usage of PreS-conjugated drugs in whole organism vaccinations, this drug also shows severe adverse side effects (see section 1.8), which could be reduced using the conjugated drug.

Before embarking on *in vivo* experiments, I initially aimed to establish the potency of PreS1-conjugated drugs in transgenic hepatocytes that constitutively express NTCP.

In this experiment, I used primaquine that had been chemically conjugated to the PreS1. Since human hepatoma cell lines do not express the PreS1 receptor NTCP, I used a transgenic hepatocyte cell line that constitutively expresses

NTCP. After infection of hepatocytes with *P. berghei* ANKA sporozoites (see section 2.3.3) liver stage parasites were exposed to 10 µM of PreS1-conjugated primaquine. 10 µM primaquine served as a positive control, 0.01% DMSO as a negative control (see section 2.3.3).

Liver stage parasites treated with primaquine show an early arrest of development. Treatment with primaquine results in smaller parasites but the same number of parasites in the liver (Putrianti et al., 2009a).

3.4.1 Analysis of *P. berghei* liver stage development under exposure to PreS1-conjugated primaquine

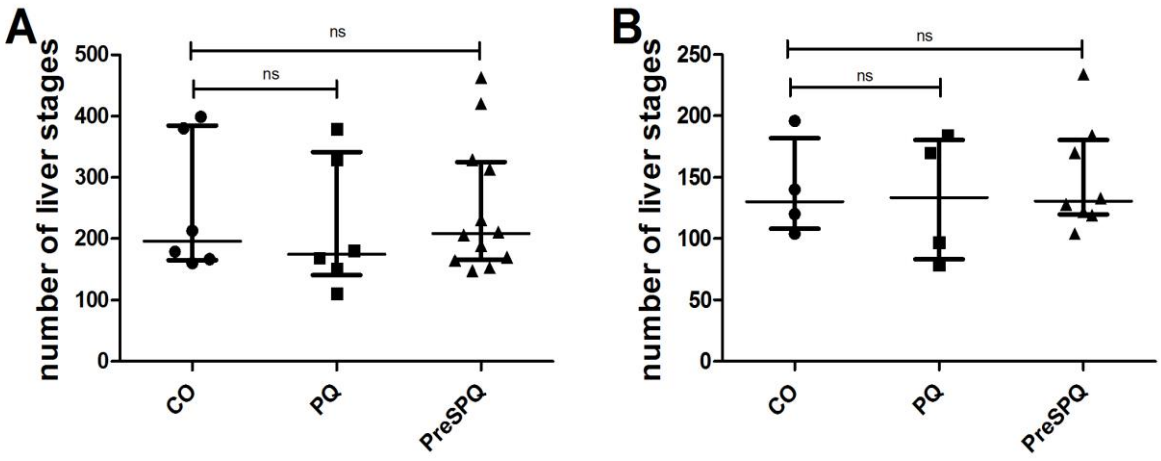


Figure 29. Treatment with primaquine or PreS1-conjugated primaquine did not affect the number of *P. berghei* liver stages. Dot plot comparing parasite numbers under different treatments. A) 48h after hepatocyte infection with following treatments: CO= 0.01% DMSO (n= 6), PQ = 10 µM primaquine (n= 6), PreSPQ= 10 µM PreS1-conjugated primaquine (n= 12). B) 65h after hepatocyte infection with following treatments: CO= 0.01% DMSO (n= 4), PQ= 10 µM primaquine (n= 4), PreSPQ= 10 µM PreS1-conjugated primaquine (n= 8). ns= non-significant, Mann-Whitney U test. Whiskers indicate median with interquartile range.

As Figure 29 shows, there was no significant difference in the overall number of liver stage parasites between control and primaquine, or control and PreS1-

conjugated primaquine treatment at 48h or 65h after hepatocyte infection. Hence, PreS1-conjugated primaquine did not affect the liver load of *P. berghei* parasites.

3.4.2 Effect of PreS1-conjugated primaquine on the size of *P. berghei* liver stage parasites

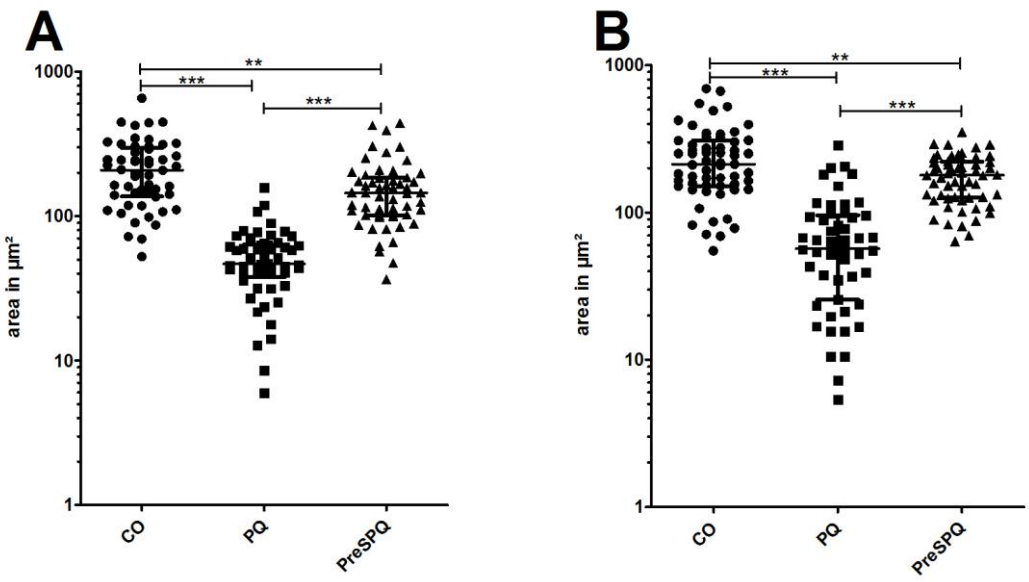


Figure 30. Both primaquine and PreS1-conjugated primaquine treatment affected the sizes of liver stage parasites but the effect was more pronounced for unconjugated primaquine. Dot plot comparing the calculated areas of *P. berghei* liver stages under different drug treatment. Each dot represents a liver stage parasite. A) After 48h of development. CO= 0.01% DMSO (n= 53), PQ= 10 µM primaquine (n= 53) PreSPQ= 10 µM PreS1- conjugated primaquine (n= 55) B) After 65h of development. CO= 0.01% DMSO (n= 59) PQ= 10 µM primaquine (n= 51) PreSPQ= 10 µM PreS1- conjugated primaquine (n=57). *** $p < 0.001$, ** $p < 0.0$, Mann-Whitney U test. Whiskers indicate median and interquartile range.

As shown in Figure 30, exposure of liver stage cultures to primaquine led to dramatically reduced sizes of parasites at both time points (early arrest). PreS1-conjugated primaquine also had an impact on the size of developed liver stage parasites. However, compared to primaquine the effect of PreS1-conjugated was much less pronounced, indicating a failure of the conjugate to release its cargo (primaquine) into the *P. berghei*-infected host cell.

4 Discussion

Probably the best instrument to contain and eventually eradicate malaria worldwide is an effective vaccination. Even though there have been several achievements to accomplish this goal there is still the need for further development.

Whole organism vaccinations using antibiotic-attenuated *Plasmodium* parasites are already known to induce sterile immunity against reinfection with the same strain. Antibiotics targeting the apicoplast, a prokaryotic organelle within the parasite, are suitable candidates (see section 1.6). However, safe and effective compounds are needed for further development. Strategies to specify the transportation of these antibiotics to the liver could additionally extend the already achieved success.

4.1 Solithromycin's mode of action against *Plasmodium spp.* regarding its usability for a whole organism vaccination

Solithromycin (characterised above, see section 1.6.2) could offer a new possibility for an antibiotic attenuated whole organism vaccine candidate due to its special chemistry and efficacy. Regarding the usability for an attenuation of *Plasmodium* parasites, I focused on certain characteristics of its mode of action.

4.1.1 Effect of solithromycin on *P. falciparum* blood stage parasites

To begin, I used the compound on *P. falciparum* blood stage merozoites. I was able to certify the effect of my compound by testing the already published IC50 and IC99 (Wittlin et al., 2012). As Wittlin *et al.* describe, solithromycin leads to a delayed death in *Plasmodium* blood stage parasites, which was the conclusion of my experiments as well. It is already known that bacteriostatic antibiotics stop the growth of *Plasmodium* parasite cultures after their second cycle of blood stage development due to their inhibition of the apicoplast's inheritance (Ramya et al., 2007).

I was also able to demonstrate that the compound did not inhibit erythrocyte invasion of merozoites. This was discussed for the macrolide azithromycin by Wilson *et al.* in 2015 (Wilson et al., 2015). However, I used the known concentrations of solithromycin for *P. falciparum* 3D7 treatment resulting in delayed death (see section 2.2.2). In the experiments of Wilson *et al*. a 760 fold higher concentration of azithromycin than typically used for the delayed death assay lead to invasion inhibition (Dahl and Rosenthal, 2007). Since I did not use these high concentrations, which probably cannot be achieved *in vivo*, I cannot exclude an inhibitory effect of solithromycin on *P. falciparum* blood stage merozoites using the experimental setup Wilson *et al.* used.

To validate the indication that solithromycin affects the prokaryotic organelle within the parasite, I used the compound on synchronised *P. falciparum* 3D7 ring stages lacking the apicoplast. As De Risi and Yeh presented, blood stage parasites only depend on the apicoplast for its IPP production (Yeh and DeRisi, 2011). Depletion of the apicoplast was achieved by treating parasites of the lab strain 3D7 with clindamycin while supplementing IPP over a certain period of time. Apicoplast free blood stage parasites are then able to survive only due to IPP supplementation.

P. falciparum parasites without apicoplast but supplemented with 100 µM IPP were not affected by solithromycin, hence showed the same development as the positive control treated with clindamycin (known to target the apicoplast) + 100 µM IPP.

In contrast to the apicoplast containing lab strain 3D7 treated with solithromycin. The treated 3D7 strain showed the typical delayed death: parasites under treatment were not able to develop further than a second cycle of erythrocyte invasion assuming an inhibition of apicoplast biogenesis (see section 1.3.2).

Since parasites lacking the apicoplast have been rescued by supplementing IPP under solithromycin treatment, I assumed an impactful effect of the compound on solely the apicoplast and no other structure within the parasite.

I also noted that the apicoplast containing 3D7 strain developed faster than typically observed (Trager and Jensen, 1976) while the apicoplast free *P. falciparum* 3D7 parasites were slightly delayed in their development, which is contrary to previous studies (Yeh and DeRisi, 2011). This could be due to the experimental condition. I used synchronised ring stage parasites as a starting point in this experimental setup. In this case, contrary to the use of merozoites as a starting point, it is very difficult to state exactly how far the different ring stages were developed. The 3D7 ring stages containing an apicoplast could have been slightly older and therefore result in faster development. Also, the fact that I used an apicoplast free parasite culture already adapted to a titrated concentration of IPP for rescuing could have played a role. I used an IPP concentration of 100µM to achieve a vivid parasite culture. However, Yeh and DeRisi showed in their paper the need for 200µM supplementation of IPP for optimal development. The concentration I used could have been enough for parasite survival but may be slightly too low for the known speed of development. Additionally, this observation could be different in further repetitions of the experiment. Since it was on main interest if the apicoplast free parasites treated with solithromycin were able to fulfil a third cycle of development in general, I did not focus on the also interesting aspect of speed of development any further.

Summarising, solithromycin affects the biogenesis and inheritance of the apicoplast of *P. falciparum* blood stage parasites in their second cycle of development. This mode of action is comparable to several other antibiotics (Dahl and Rosenthal, 2007).

4.1.2 Effect of solithromycin on *P. berghei* liver stage parasites

To continue my investigations, I focused on an earlier stage of the parasite's life cycle. I used *P. berghei*, a rodent malaria parasite, to explore the liver stage of *Plasmodium* parasites. The aim of this study is to explore the effect of solithromycin on the apicoplast in *Plasmodium* parasites. Since host-parasite interaction does not play a role but solely the biological effect of the antibiotic on the parasite and especially its prokaryotic organelle, findings in *P. berghei* can be transferred easily to human pathogen *Plasmodium* species.

Demonstrated in murine models by Butler *et al.*, late liver stage arrested parasites led to better protection against reinfection than earlier elimination of the parasite from the liver due to enlarged $CD8^+$ t cell reaction (Butler et al., 2011). Therefore, I focused on the effect of solithromycin regarding the timepoint of liver stage arrest and its target within parasites.

The first stage of the parasites' lifecycle, sporozoites and their entering of the liver parenchyma, was also the first object of interest. Incubation with solithromycin prior hepatocyte invasion did neither affect the invasion rate of treated sporozoites, nor the total number of developed liver stage parasites after 65 hours compared to the control group. Hence, solithromycin does not stop the parasite's development at this early point but allows further development in the liver and duration of infection, consequently further antigen presentation to the host's immune system. Like already mentioned, comparable to the effect of azithromycin and chloroquine (see section 1.6).

In comparison to the following experiments using not preincubated sporozoites, the overall liver load of the invasion assay was lower than without incubation of sporozoites before entering the liver. Since the fragile sporozoites were incubated with the compound for one hour before they were added to the Huh7 hepatoma cells, there certainly was a partial loss of sporozoites in this time interval. However, since the amount of invaded pre-incubated sporozoites in the control group was lower as well, the compound did not show an inhibiting effect on invasion.

Following the parasites' life cycle, I monitored solithromycin's effect on *P. berghei* liver stage parasites. Azithromycin was used as a positive control. Previous studies showed that an increased liver load (which is also related to the application of parasites) correlates with better protection against reinfection using whole organism vaccinations (Nganou-Makamdop et al., 2012a).

None of the antibiotic treatments influenced the overall number of liver stages after both, 48 and 65 hours. However, some outliers appeared which were due to experimental repetition. In every experiment, different sporozoites dissected from various *Anopheles* mosquitos were used. Some seem to be more potent in

invasion than others. Not only biological individuality but also the difference between several repetitions of the experiment regarding processing time of sporozoites from dissection out of mosquitos until invasion of hepatocytes could play a role.

Giving particular attention to the measured sizes of *P. berghei* liver stage parasites, a new aspect was presented: DMSO concentration. Azithromycin was solved in distilled H_2O. But since solithromycin was solved in DMSO, I chose the concentration of DMSO in the control group adaptively to the concentration of the chemical in the solithromycin group, which was eventually a concentration of 1 % DMSO.

With this experimental setup, I observed an inconsistent effect of the two antibiotics used: Parasites treated with azithromycin showed no defect in growth after 48 hours but after 65 hours of development. This was opposed to treatment with solithromycin which showed a defect of growth after 48 hours but not after 65 hours of development. Different possible reasons for this observation exist: 1) solithromycin could solely lead to a delay in the growth of parasites in the liver while not affecting the total number or the final size. 2) As already published, azithromycin targets the apicoplast within the liver stage parasite (Friesen et al., 2010). As described before (see section 1.3), this organelle assumes a branching structure while distribution to developed daughter cells in a liver schizont. Lacking this organelle and its expansion, parasites could be affected in their size. However, solithromycin is strongly expected to use the apicoplast as a target as well, hence should lead to a decrease of size as well. 3) As mentioned previously (see section 3.2), DMSO has different adverse effects. In comparison to solithromycin, azithromycin was solved in solely distilled H_2O and therefore azithromycin treated parasites were not influenced by DMSO. Solithromycin however, solved in DMSO, is a substrate of cytochrome P 3A4 which is induced by DMSO (Still et al., 2011). Induction of cytochrome could lead to a faster elimination of solithromycin within the cells and therefore a faster decrease of concentration. Additionally, DMSO intensifies cytotoxic effects. These aspects could provide an explanation for diverse data in the control and solithromycin group.

In order to counter the bias, I changed the concentration of DMSO into 0.01%. I additionally applied a lower concentration of solithromycin with regard to cytotoxicity. Even with a decreased concentration of DMSO, treatment with 10 µM solithromycin led to significantly smaller liver stage parasites after 48 hours of development in contrast to treatment with azithromycin and 1 µM solithromycin. Cytotoxicity of 10 µM solithromycin could result this outcome by targeting not only the apicoplast but also other structures of the liver stage parasite in this high concentration. However, to date, there are no published data about the compound's hepatocytotoxicity *in vitro*. Yet, solithromycin is not approved because the FDA criticises the lack of evidence of its clinical safety regarding possible liver toxicity *in vivo* likewise its predecessor telithromycin (Abdulla, 2016). However, in phase III clinical trials the compound was declared to be safe and telithromycin's adverse side effects were attributed to a pyridine side chain (see section 1.6.2) which does not exist in solithromycin's configuration (Fernandes et al., 2016, Jamieson et al., 2015). Nevertheless, further experiments concerning cytotoxicity *in vitro* and *vivo* are indispensable.

Having a look at the later time point under these conditions, every treatment was followed by development of significantly smaller liver stage parasites, even the parasites treated with solithromycin 10µM, in contrast to the experimental set up with DMSO 1%. This could again be explained by the cytochrome induction of DMSO in a higher concentration and the lack of the apicoplast due to treatment with azithromycin as well as solithromycin.

Since I monitored different results of matured sizes of *P. berghei* liver stage parasites by changing the concentration of DMSO, I compared the different experimental conditions concerning the DMSO concentration and appearance of developed parasites. Higher concentration of DMSO did not affect the overall number of parasites developed in the liver. The occurred sizes varied very inhomogeneously as described and discussed above. However, since azithromycin was not solved in DMSO but in distilled H_2O and showed significant differences of sizes in the compared experiments as well, it seems reasonable to suppose that not solely the concentration of DMSO influenced the sizes of liver stages but also other logistical reasons. Individuality of parasites and HuH7 cells,

processing time, measurement errors plus experimental repetition as an independent variable could have played a role.

However, with respect to the median of parasites' sizes, the difference between the control group and azithromycin or 1 µM solithromycin treated parasites are respectively approximately around 50 µm², between the control group and parasites treated with10 µM solithromycin approximately around 80 µm².

Table 5. Comparison of medians of liver stage parasites' sizes after 65h of development

TREATMENT	MEDIAN	DIFFERENCE TO CONTROL
Control (0.01% DMSO)	267.3 µm²	
Azithromycin	215.6 µm²	51.7 µm² (- 19%)
Solithromycin 1 µM	216.1 µm²	51.2 µm² (-19 %)
Solithromycin 10 µM	187.0 µm²	80.3 µm² (- 30%)

The presented differences of measured sizes are, considering biology, of no significant consequence regarding antigen presentation hence a whole organism vaccination. Especially because parasites are alive and metabolic active. Though further investigations of presented antigens while development, like the extensively studied SpzCSP, might lead to other interesting insights (Kumar et al., 2006).

Demonstrated before, solithromycin targets the apicoplast in *P. falciparum* blood stage parasites. Relating to my interest of solithromycin inhibiting the apicoplast in liver stage parasites likewise, I presented via immunofluorescence assay the lack of the branched organelle in solithromycin treated liver stage parasites after 65 hours of development. There was no apicoplast distribution to daughter cells. At this point, solithromycin's mode of action on *Plasmodium* parasites is similar to azithromycin's effect (Friesen et al., 2010). This underlines its abilities as a candidate for the chemical attenuation of parasites.

4.1.3 Solithromycin's effect on the infectivity of *P. berghei* liver stage merozoites

Whereas DMSO concentration of 1% did not show a stable effect on liver stage parasites, it completely inhibited the emerge of merosomes out of hepatocytes. Solithromycin treated sporozoites developed to liver stage parasites within 65 hours with no difference in appearance compared to the control group. Hence these parasites represent metabolic active cells. There was exclusively a defect in emerging merosomes in the wells' supernatant, which is to date not described in literature. This finding could be due to DMSO's effect of loosening cell membranes (de Menorval et al., 2012). The parasite requires the host hepatocyte to build up merosomes, the membrane of these daughter cell packed vesicles consists of the host's cell membrane (Sturm et al., 2006). Hence, the parasite might not be able to build up the merosome membrane under the influence of high concentrations of DMSO.

Accompanying this information, I changed the concentration of DMSO from 1% to 0.01%. The decrease of concentration led to an increase of merosome appearance in the control group. However, I was able to show the altering effect of solithromycin on merosome formation and occurred concentration. Moreover, merosomes emerging from treated parasites were not infective. After i.v. injection into mice, merosomes emerging from solithromycin treated parasites were not able to infect erythrocytes and thus did not lead to parasitaemia.

Since the experimental setup and harvesting of merosomes under treatment condition was accompanied by a lower concentration of occurred merosomes, I was not able to inject the exact same number of parasites into mice of the two groups. In my first experiment, I injected a lower number of solithromycin treated parasites (all merosomes out of 8 wells, 210 respectively) into mice than into the control group (all merosomes out of 8 wells, 410 respectively) which could also explain parasitaemia free animals. However, much less numbers of merosomes (100) were shown to result in blood stage infection after using the same injection strategies I used (Friesen et al., 2010). Hence, the injection of 210 merosomes should lead to a blood stage infection if merosomes were infectious. Moreover, I

was able to inject approximately the same amount of merosomes in both groups in a repetition experiment resulting in the same outcome.

There are several modifications or knock-outs of genes of the parasite which are proven to change merosome formation regarding number or delay in occurrence of merosomes. Some of these affected structures are located in the parasitophorous vacuole, like a phospholipase (PbPL), serine kinases or the protein EXP-1. There are also enzymes associated with merozoites' nuclei like the serine kinase SUB1 (Burda et al., 2015, Suarez et al., 2013).

Additionally, and in this case much more interesting, there are relevant proteins of the apicoplast, of which knockouts lead to fewer or even lack of merosomes, close to what I observed under solithromycin treatment. These proteins are widely spread in the different sections of the apicoplast's functions:

For example, there is NFUapi, which is crucial in an iron-sulfur cluster or PALM which plays an important role in only liver stage parasites but does not influences apicoplast segregation (Haussig et al., 2011, Haussig et al., 2013). Moreover, a protein called Fabl in the fatty-acid pathway and a liver specific protein called LISP2 which is exported into the cytoplasm of the hepatocyte for merozoite formation play an important role (Orito et al., 2013, Yu et al., 2008).

The loss of these proteins results in a significantly lower number of occurred merosomes. However, the merozoites emerging are infective, which means they are able to invade erythrocytes and cause malaria symptoms. This is in contrast to my results, showing that solithromycin treated merosomes were not infective, likewise, merosomes emerging from azithromycin treated parasites (Friesen et al., 2010). Additionally, the observance of solithromycin resulting in delayed death in blood stage parasite assumes targeting of housekeeping functions and no influence on metabolic pathways within the apicoplast.

To date, one cannot find a description of antibiotics leading to a decrease in the number of formed merosomes in literature. Additionally, there is no reference of a treatment causing structural deficits of merosomes. My basic observance

certainly demands further experiments focusing on the structural deficits and decreased number of occurred merosomes.

Still, concerning biological consequences, emerging of uncontagious merosomes as a result of solithromycin treatment is exactly what is asked for a CAS vaccination approach. However, I neither investigated the immediate response of the rodents' immune system, nor the probably build immunity after injection of uninfectious *P. berghei* merosomes. But since the requested effect was shown after the same experimental setup for azithromycin, it can be assumed that inoculation of solithromycin treated merosomes result in protection against reinfection with the same strain as well (Friesen et al., 2010).

4.2 Effect of PreS1-primaquine on *P. berghei* hNTCP Huh7 liver stage parasites

The second aim of this study was to test PreS1-conjugated primaquine on *P. berghei* liver stages to verify or disprove a liver specific effect of the compound due to distinct transportation to the liver.

Described above (see section 1.7.2) the preS1 surface protein of the hepatitis B virus was already used for liver specific transportation. Stephan Urban's working group established and kindly provided a peptide conjugation of PreS1 and primaquine, to test whether this liaison could lead to liver specific delivery of the antimalarial compound. Hence, enhance the effect of primaquine on liver stage parasites while reducing the systemic adverse effects. Thus, I compared unconjugated vs. conjugated primaquine with respect to its impact on the development of *P. berghei* liver stage parasites.

Primaquine's mode of action, eliminating or rather causing nonviable liver stage parasites, was already described by Putrianti *et al.* and confirmed my results (Putrianti et al., 2009b). The liver load of parasites in the two treatment groups did not differ from the control however there was a difference in parasites' sizes in data of both measured time points as described above (see section 3.4).

Table 6. Comparison of medians of liver stage parasites' sizes after 48h of development

TREATMENT	MEDIAN	DIFFERENCE TO CONTROL
Control	208.0 µm²	
Primaquine	46.56 µm²	162,44 µm² (- 77,65%)
PreS1-primaquine	145.1 µm²	62,9 µm² (- 30%)

Table 7. Comparison of medians of liver stage parasites' sizes after 65h of development

TREATMENT	MEDIAN	DIFFERENCE TO CONTROL
Control	212.7 µm²	
Primaquine	56.0 µm²	156 µm² (- 73,7 %)
PreS1-primaquine	179.7 µm²	32,3 µm² (-16 %)

As shown in Table 6 and 7, the smallest parasites were measured after treatment with primaquine, the differences of 162,44 µm² after 48 hours of development and 156 µm² after 65 hours demonstrate a strong impact on the maturation of liver stage parasites. Primaquine treatment reduced the area of parasites by approximately 75%.

In contrast to this observation, even though the treatment with PreS1-conjugated primaquine reduced the measured sizes of liver stages as well, there was a minor impact. Sizes were reduced by 30% at the earlier time point and even less (16%) after 65 hours of liver stage development. As described above (see section 3.2), differences in size followed by PreS1-conjugated primaquine treatment might not play a role regarding biology and assume that viable parasites remained after treatment.

I expected the conjugation to show an equal or even enhanced effect of primaquine but unfortunately, the peptide conjugation of PreS1 and the antimalarial drug did not show the expected effect. Thus, did not lead to a liver

specific transport of primaquine, in contrast to the already published effect of a conjugation with pegylated liposomes and lipid nanoparticles (see section 1.7.2).

This observation suggests that the peptide conjugation of Pres1 and primaquine is too strong, hence the compound is not able to loosen from the peptide and so remains outside hepatocytes. Since the NTCP receptor is saturated fully with much less than a concentration of 10µM which I used in my experiments, I would not expect any effect even with higher concentrations (Watashi et al., 2014b). Therefore, I promote further experiments based on e.g. lipid nanoparticles.

4.3 Conclusion

To conclude, the presented results of solithromycin's effect on *Plasmodium* parasites indicate the compound as an eligible candidate for a whole organism vaccination. With the apicoplast as a target, it allows liver stage parasites to develop into uninfectious merosomes to achieve a long antigen presentation to the host's immune system without causing malaria symptoms. However, there is an urgent need for further studies on its safety. Furthermore, detailed investigations regarding the immune response of rodents after immunisation with solithromycin treated merosomes must be performed. Additionally, trials focusing on the induced immunity by antibiotic attenuated parasites, especially regarding cross or strain specific immunity are necessary to be able to further pursue this approach.

Unfortunately, the peptide PreS1-conjugation of primaquine did not show the expected effect on *P. berghei* liver stage parasites. More promising approaches e.g. based on lipid nanoparticles have to be investigated further.

5 Summary

Despite successful efforts to decrease suffering from malaria, the impact on global health still is enormous. Prevention is a key element in the fight against this parasitic disease. Malaria might only be contained and eventually eliminated worldwide by a vaccination that is affordable, safe and efficient. Whole organism vaccinations using antibiotic-attenuated parasites feature these potentials.

This study comprises two different aspects. Firstly, the main interest was on peptide-conjugated drugs. A conjugation of drugs with the hepatitis B virus' large surface antigen (PreS1), which constitutes the virus' entry key to hepatocytes was tested. Not only could this conjugation be applied in one single shot with the parasites and thus simplify the application process of the vaccine. It could also lead to a specific transport of drugs to the liver and consequently result in a reduction in dose and minimisation of adverse side effects. PreS-conjugation with a well-established antimalarial, primaquine, was tested on *Plasmodium* liver stage parasites. Unfortunately, treatment with the drug conjugation did not show the wished-for effect and therefore needs further investigations.

Secondly, the mode of action of the new fluoroketolid antibiotic solithromycin regarding the possible usage in a whole organism vaccination was investigated. The compound showed an effect on the apicoplast in blood and liver stage parasites. It could be demonstrated that solithromycin inhibits the inheritance and segregation of this prokaryotic organelle into the parasites' daughter cells. Experiments with blood stage parasites showed the typical "delayed death" phenomenon. Treated liver stage parasites were arrested in a late stage of development, hence could induce long and various antigen stimulation of the host's immune system. However, i.v. injection of treated liver stage merosomes in mice revealed that treated daughter cells emerging from the liver are not able to invade erythrocytes. Consequently, there would be no incident of malaria symptoms but only the establishment of protection against reinfection.

The findings suggest that solithromycin is a suitable drug for attenuation of *Plasmodium* parasites. Therefore, I promote further investigations e.g. regarding

safety, immunostimulation after i.v. merosome injection and subsequently challenging with viable parasites for a further development of whole organism vaccinations against malaria.

Zusammenfassung

Trotz erfolgreicher Bemühungen, das Leiden an Malaria zu mindern, sind die Auswirkungen auf die globale Gesundheit immer noch enorm. Prävention ist ein Schlüsselelement im Kampf gegen diese parasitäre Krankheit. Malaria kann möglicherweise nur durch eine Impfung, die erschwinglich, sicher und effizient ist, eingedämmt und letztendlich weltweit eradiziert werden. Impfungen mit lebenden Parasiten, die mit Antibiotika abgeschwächt wurden, bieten diese Möglichkeiten.
Diese Studie umfasst zwei verschiedene Aspekte.

Als erstes lag das Interesse auf peptidkonjugierten Arzneimitteln. Es wurde eine Konjugation von Arzneimitteln mit dem großen Oberflächenantigen des Hepatitis-B-Virus‘ (PreS1) getestet, das den Eintrittspunkt des Virus in Hepatozyten darstellt. Diese Konjugation könnte nicht nur zeitgleich als Einzelgabe mit den Parasiten appliziert werden und so den Verabreichungsprozess vereinfachen. Es könnte ebenfalls zu einem spezifischen Transport von Arzneimitteln in die Leber führen und folglich zu einer Dosisreduzierung und Minimierung von Nebenwirkungen führen. Die PreS-Konjugation mit einem bewährten Malariamittel, Primaquin, wurde an Plasmodiumparasiten im Leberstadium getestet. Die Behandlung mit der Wirkstoffkonjugation zeigte leider nicht den gewünschten Effekt und bedarf daher weiterer Untersuchungen.
Als zweiter Punkt wurde die Wirkungsweise des neuen Fluorketolid-Antibiotikums Solithromycin im Hinblick auf die mögliche Anwendung innerhalb einer Impfung mit attenuierten Parasiten getestet. Der Wirkstoff zeigte einen Effekt auf den Apikoplasten der Parasiten im Blut- und Leberstadium. Es konnte gezeigt werden, dass Solithromycin die Vererbung und Segregation dieser prokaryotischen Organelle in die Tochterzellen der Parasiten hemmt.

Experimente mit Parasiten im Blutstadium zeigten das typische Phänomen des sogenannten „delayed death“. Behandelte Parasiten im Leberstadium wurden in einem späten Stadium in ihrer Entwicklung gestoppt und könnten daher eine lange und diverse Antigenstimulation des Immunsystems des Wirts auslösen. Die intravenöse Injektion von behandelten Leberstadien-Merosomen in Mäuse ergab, dass behandelte Tochterzellen, die aus der Leber austreten, nicht fähig sind Erythrozyten zu befallen. Infolgedessen würden keine Malariasymptome auftreten, sondern lediglich ein Schutz gegen eine erneute Infektion geschaffen. Die Ergebnisse legen nahe, dass Solithromycin ein geeignetes Medikament zur Abschwächung von Plasmodium-Parasiten ist. Daher befürworte ich weitere Untersuchungen, z.B. in Bezug auf Sicherheit, Immunstimulation nach i.v. Merosom-eninjektion und zuletzt Überprüfung des Schutzes mittels lebenden Parasiten, für die Weiterentwicklung einer Impfung gegen Malaria.

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Ich versichere das Manuskript eigenhändig verfasst zu haben und keine weiteren Quellen als die von mir angegebenen Quellen verwendet zu haben.

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