Aus der

Medizinischen Universitätsklinik und Poliklinik Tübingen
Abteilung VII, Tropenmedizin

(Schwerpunkt: Institut für Tropenmedizin, Reisemedizin, Humanparasitologie)

In vitro activity of antibiotics against Plasmodium falciparum and Plasmodium knowlesi

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Medizin

der Medizinischen Fakultät

der Eberhard Karls Universität

zu Tübingen

vorgelegt von

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2025

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Tag der Disputation:	22.08.2025

Dedication

For all malaria patients and all scientists working on the control of this disease

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1 Introduction

1.1 Malaria

1.1.1 The disease

Malaria is a tropical disease caused by *Plasmodium* species and transmitted by the female *Anopheles* mosquito. It remains one of the leading causes of morbidity and mortality worldwide. According to the 2023 World Health Organization report, an estimated 249 million malaria cases and 631,000 deaths occurred in 2022, with children under five years of age accounting for 76% of deaths (1). From 2000 to 2022, Sub-Saharan Africa bore a disproportionate burden of malaria, accounting for 82% of cases and 94% of deaths, followed by Southeast Asia with 10% of cases and 3% of deaths (1).

The clinical manifestation and prognosis of malaria are heavily influenced by the infecting *Plasmodium* species, which include six human pathogens: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (both *P. ovale curtisi* and *P. ovale wallikeri*), and *P. knowlesi (2)*. All species present with nonspecific symptoms such as fever, chills, sweating, headache, weakness, myalgia, nausea, and vomiting following a brief prodromal phase (2). The febrile paroxysm correlates with the rupture of the schizonts, and thus depends on the duration of the erythrocytic cycle (see section 1.1.2, *Plasmodium* spp. life cycle) (3). *P. knowlesi* malaria is characterized by a daily fever (approximately 24-hour life cycle), whereas *P. vivax* and *P. ovale* infections exhibit a tertian fever pattern (fever paroxysms every 48 hours) due to schizont rupture every 48 hours (4, 5). *P. malariae* causes fever paroxysms every 72 hours (malaria quartana) (4, 5). Although the life cycle of *P. falciparum* lasts approximately 48 hours, the fever paroxysms typically occur every 24 hours due to asynchronous parasite population growth (3, 6).

Malaria can be classified as asymptomatic, uncomplicated, or severe. Asymptomatic malaria is defined by circulating parasites in individuals who exhibit no symptoms. Uncomplicated malaria refers to symptomatic individuals in whom parasitaemia has been detected via microscopy or rapid diagnostic tests

but who do not meet the criteria for severe malaria. Severe malaria is defined by the WHO as an infected person with one or more of the following clinical or laboratory findings (7).

Clinical manifestations:

- Impaired consciousness
- Respiratory distress (acidotic breathing)
- Multiple convulsions
- Prostration
- Shock
- Pulmonary oedema
- Abnormal bleeding
- Jaundice

Laboratory findings:

- Severe anaemia (hemoglobin < 50 g/L or hematocrit < 15%)
- Hypoglycaemia
- Acidosis
- Hyperlactataemia
- Acute kidney injury
- Hyperparasitaemia

P. falciparum is the most prevalent species globally and is responsible for the majority of severe malaria cases. In the most affected region, Sub-Saharan Africa, it accounted for 99.7 % of estimated malaria cases in 2018 (8).

P. knowlesi, the leading cause of human malaria in Malaysia *(9, 10),* is geographically restricted to Southeast Asia, following the distribution of its natural hosts: the long-tailed macaque (*Macaca fascicularis*), the pig-tailed macaque (*Macaca nemestrina*) and the banded-leaf monkey (*Presbytis melalophos*) (11, 12). In 2004 *P. knowlesi* was formally recognized as a clinically and epidemiologically relevant human malaria parasite (13). Like malaria tropica, *P. knowlesi* malaria can present as either uncomplicated or severe, though it

typically poses a lower individual and epidemiological disease burden compared to *P. falciparum*.

1.1.2 Plasmodium spp. life cycle

Plasmodium spp. are obligate intracellular parasites with a complex life cycle that alternates between an invertebrate vector (*Anopheles* mosquito) and a vertebrate host (such as humans). This life cycle comprises three major stages: the human exo-erythrocytic (hepatic) stage, the human erythrocytic stage, and the mosquito sporogonic stage (Figure 1).

During a blood meal, female *Anopheles* mosquitoes inject sporozoites into the human bloodstream via their saliva. These sporozoites rapidly disseminate via the circulatory system and invade hepatocytes, thereby initiating the exo-erythrocytic (liver) stage of development ([1] in Figure 1) (14). Within hepatocytes, sporozoites differentiate into liver-stage schizonts ([2] in Figure 1) (15). Upon maturation, the schizonts give rise to merozoite-containing vesicles, termed merosomes, which bud off from the host cell and release thousands of merozoites into the bloodstream ([3] in Figure 1). These merozoites invade erythrocytes, marking the beginning of the asexual intraerythrocytic replication cycle ([4] in Figure 1).

Inside erythrocytes, the merozoites sequentially develop into ring forms ([5]), trophozoites ([6]), and finally mature schizonts ([7]) (Figure 1), which rupture the host cell and release new merozoites that reinvade erythrocytes to sustain the erythrocytic cycle (5). A subset of ring-stage parasites differentiates into gametocytes ([8] in Figure 1), the sexual forms of the parasite. Gametocytogenesis progresses through five morphological stages (I–V), with immature forms (stages I–IV) sequestered in the bone marrow. Only mature stage V gametocytes circulate in peripheral blood, where they can be taken up by a feeding mosquito, thus perpetuating the life cycle (5, 16).

The mosquito-stage of the *Plasmodium* life cycle, known as sporogony, represents the sexual phase and includes meiotic events. In the mosquito midgut, male microgametocytes undergo exflagellation and fertilize female macrogametocytes, resulting in the formation of diploid zygotes ([9] in Figure 1).

These zygotes elongate and transform into motile ookinetes ([10]), which traverse the midgut epithelium and form oocysts beneath the basal lamina ([11]). Upon maturation, oocysts rupture and release thousands of sporozoites ([12]) that migrate to the salivary glands, ready for transmission to a new human host during a subsequent blood meal (17).

P. vivax and *P.* ovale have a unique ability to form dormant liver-stage parasites known as hypnozoites. These forms can persist in hepatocytes for weeks to years without causing clinical symptoms. Upon reactivation, hypnozoites resume replication, re-enter the bloodstream as merozoites, and initiate the erythrocytic cycle, leading to relapse of malaria (18, 19).

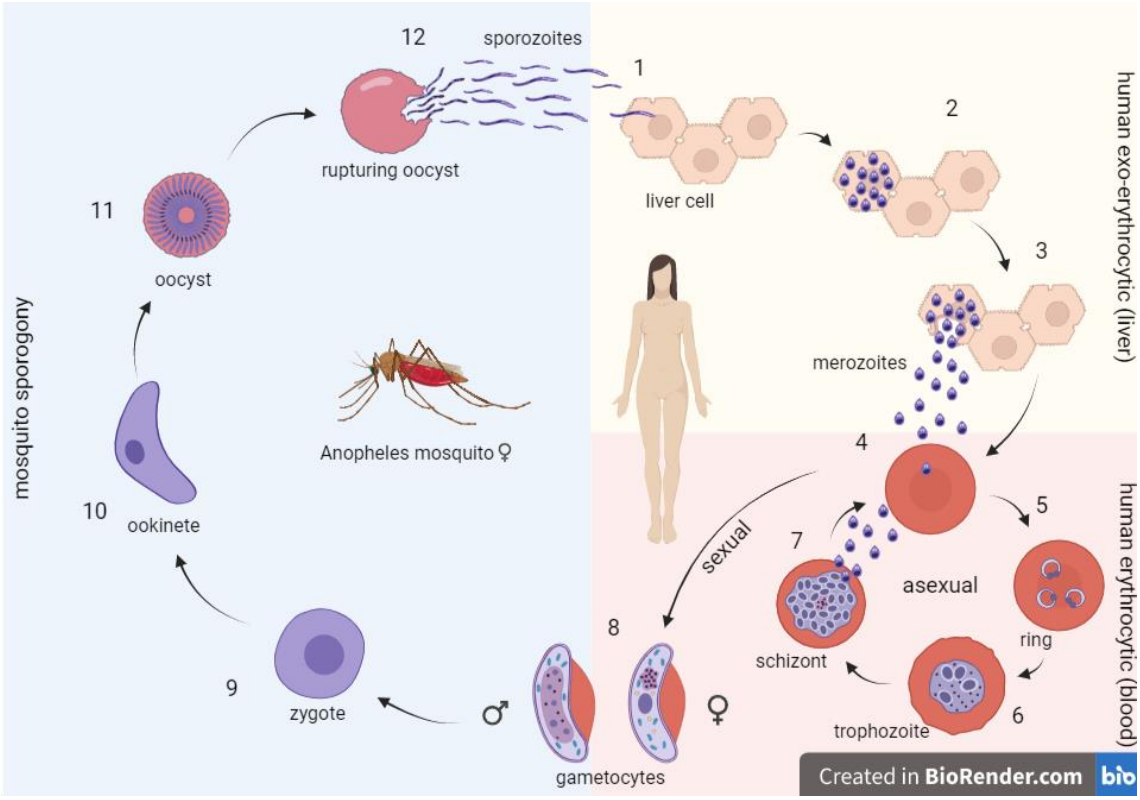

Figure 1 Malaria life cycle: This figure illustrates the stages of the Plasmodium life cycle in both the human host and the mosquito vector. [1-3] Development of merozoites within human liver cells; [4-7] Asexual replication in human red blood cells, encompassing the invasion of merozoites, as well as the formation of rings, trophozoites, and schizonts; [8] Development of male and female gametocytes in the human host, which are ingested by the mosquito; [9-12] Sporogony in the mosquito, where fertilized gametocytes mature into zygotes, ookinetes, oocysts, which subsequently rupture to release sporozoites.

1.1.3 The apicoplast

The apicoplast is a relict non-photosynthetic plastid of algal origin, acquired through secondary endosymbiosis, and plays an essential role in *Plasmodium spp.* (20-22). The apicoplast contains its own circular genome (~35 kb), encoding ~30 proteins, tRNAs, and bacterial-type ribosomes, highlighting its semi-autonomous nature (23). Nonetheless, the majority of its proteins are encoded in the nucleus and imported into the organelle. (21, 24).

The apicoplast still harbours some prokaryotic metabolic pathways that are indispensable to the parasite throughout its life cycle (Figure 2) as confirmed by *Yeh and DeRisi* (25). Furthermore, its genome is well conserved within the different *Plasmodium* species as well as in *Apicomplexa* genus, providing an interesting drug target to which multiple pathogens may be susceptible (21). Moreover, due to its bacterial-like ancestry, it is possible to develop compounds that target this structure itself and thus have low toxicity to the host (21).

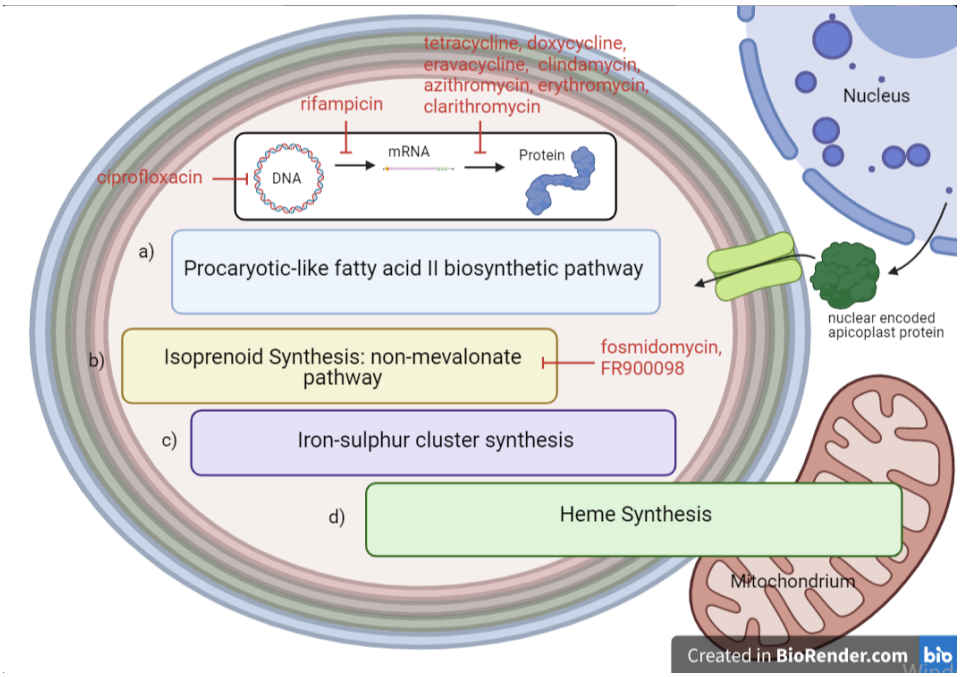

Figure 2 P. falciparum´s apicoplast´s five central metabolic pathways: This figure outlines the five key metabolic pathways within the apicoplast of Plasmodium falciparum: protein biosynthesis based on the apicoplast's own DNA, prokaryotic-like fatty acid II biosynthesis, isoprenoid synthesis, iron-sulfur cluster synthesis, and heme synthesis. Notably, heme synthesis occurs both in the apicoplast and the mitochondrion. Antibiotics and their respective sites of action within the apicoplast, which will be discussed in subsequent chapters, are highlighted in red. Additionally, the figure illustrates that the apicoplast not only relies on proteins encoded by its own genome but also requires nuclear-encoded apicoplast proteins that are imported into the organelle.

a) Prokaryotic-like fatty acid II biosynthesis pathway (FAS II)

Plasmodium parasites acquire fatty acids from their hosts or by building *de novo* via the FAS II. In contrast to cytosolic type I FAS housed in eukaryotes and working with a single multifunctional enzyme, FAS II pathway has its origin in prokaryotes and functions with various enzymes (26).

The deletion of different enzymes of the anabolic process of *Plasmodium* spp. revealed, that the absence of FAS II does not affect replication of parasites in blood stage but is critical to the development of late liver stage parasites (26, 27). Previous research has proposed that the fatty acids are needed for the formation of the parasitophorous vacuole, which envelops the intracellular parasite, protects it from destruction by the host cell and is built by every invading merozoite (28). In the liver stage, each sporozoite produces thousands of merozoites while in the blood stage one schizont only produces about 20 merozoites (29). This difference may explain the indispensability of FAS II for the late liver stage (24, 30).

b) Isoprenoid synthesis

Isoprenoids consist of lined up isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate, and their synthesis is the only essential apicoplast pathway in blood stage parasites (25). Isoprenoids have different vital functions by being involved in electron transport, glycoprotein formation and acting as prosthetic groups in various enzymes (25). The literature about isoprenoid synthesis reveals two different biosynthesis pathways: the mevalonate pathway occurring in eukaryotes and the non-mevalonate pathway (MEP/DOXP) harboured in bacteria and plastids. The apicoplast houses the MEP/DOXP pathway, thus there is essentially no commonality between the parasite isoprenoid synthesis and the human isoprenoid synthesis, making MEP/DOXP a promising drug target.

Yeh and DeRisi (25) developed a functional assay to discriminate apicoplast-targeting drugs: parasites treated with a compound are supplemented with IPP; if growth is rescued, the compound targets the apicoplast. If no rescue occurs, the apicoplast can be excluded as the primary target.

Most antibiotics that affect the apicoplast interfere with housekeeping functions such as transcription and translation, leading to gradual organelle loss. Uddin et al. (31) demonstrated that such disruption results in genome loss, impaired protein import, and morphological abnormalities. These effects typically manifest as the "delayed death" phenotype - defined by the absence of drug activity in the first life cycle, followed by pronounced inhibition in the second cycle. This is attributed to daughter cells inheriting a non-functional or absent apicoplast, while the parent cell had sufficient pre-existing enzymes to support initial development (25). In contrast, fast-acting drugs such as fosmidomycin and its derivative FR900098 directly inhibit isoprenoid biosynthesis by targeting DXP reductoisomerase. These compounds do not interfere with apicoplast maintenance, and their antiparasitic activity becomes evident during the first cycle. The figure below outlines the standard approach for classifying antibiotics based on their onset of action, distinguishing between fast- and delayed-death mechanisms.

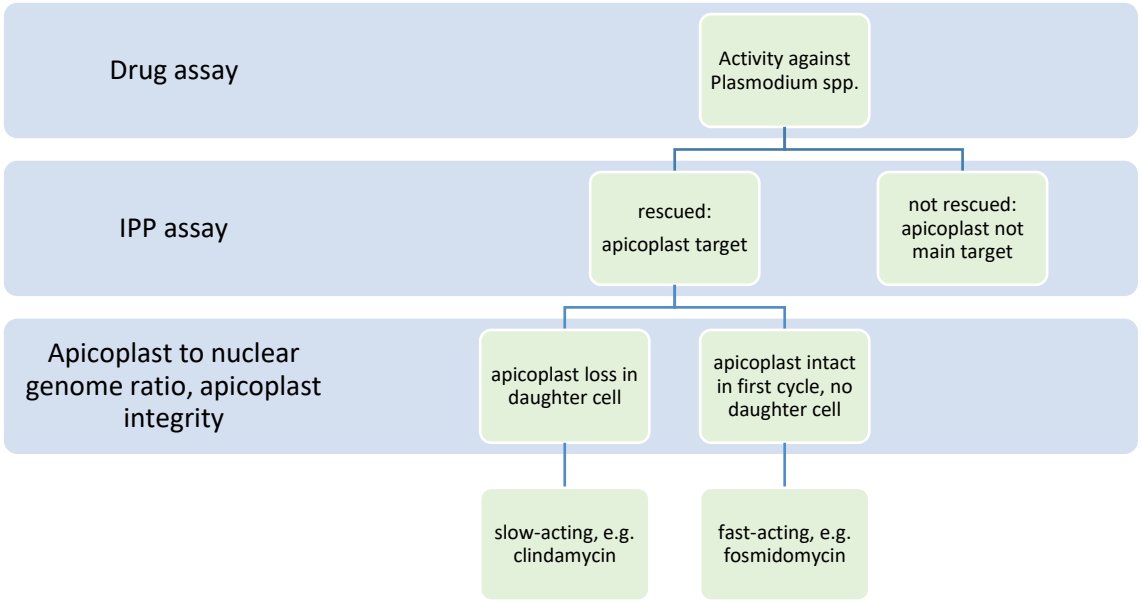

Figure 3 Procedure for classification of apicoplast-targeting drugs in P. falciparum. This figure illustrates the workflow for classifying drugs targeting the apicoplast in Plasmodium falciparum. If in vitro drug activity is confirmed in a drug assay, an additional assay with IPP supplementation is performed. If parasite rescue occurs upon IPP addition, the apicoplast/nuclear genome ratio is determined to assess whether a daughter cell without an apicoplast is produced, as seen with slow-acting drugs, or whether no daughter cell is generated at all, as observed with fast-acting drugs.

c) Iron-sulphur cluster formation

Another ability of the apicoplast is the formation of iron-sulphur clusters, which are typically cofactors at enzymatic reactions. Normally, the demand is covered by the cluster synthesis in the mitochondrion and nucleus, but this activity was also discovered in the apicoplast. The enzymes for iron-sulphur cluster synthesis in the apicoplast are encoded partly in the apicoplast (SufB, Orf470) and partly in the nucleus (NifU, SufA, SufC, SufD, SufS). So far, however, no drugs inhibiting this pathway are available, and it is also not essential for parasite survival in the erythrocytic cycle (30).

d) Heme biosynthesis

The fourth function of the apicoplast is the heme biosynthesis (25). In the parasite, two heme metabolic pathways can be distinguished: on the one hand, the degradation of the host cell's heme and, on the other hand, the *de novo* synthesis of the parasite's own heme (32, 33). Since there is enough heme available in erythrocytic stage, the need for *de novo* synthesis of heme is especially relevant in extra-erythrocytic stages. Consequently, it was hypothesized, just like fatty acid and iron-sulphur cluster synthesis, heme biosynthesis, but not heme existence, is dispensable in the erythrocytic stage. This anabolic pathway was of special interest as it was described as the drug target of herbicides and succinylacetone (24, 31) but recently, drug activity of succinylacetone in the blood stage was related to off-target activity (32). Inhibition of heme synthesis could be interesting to study as a method to reduce transmission of malaria as heme biosynthesis is indispensable for mature male gametocyte formation and maturation within the mosquito (32).

1.1.4 Current therapy guidelines 2022

According to WHO guidelines of 2022 (34) the gold standard of malaria therapy in endemic areas is based on artemisinin combination therapy (ACT). This is composed of a fast-acting artemisinin derivative, which can strongly reduce the parasitaemia and a slow-acting partner drug which eliminates the remaining parasites in the blood stream and may prevent resistance development to the artemisinin derivative. The six recommended combinations are:

- artemether + lumefantrine
- artesunate + amodiaquine
- artesunate + mefloquine
- dihydroartemisinin + piperaquine
- artesunate + sulfadoxine-pyrimethamine
- artesunate + pyronaridine

In addition to antimalarial treatment, symptomatic therapy can be given; most importantly, glucose for hypoglycemia and blood transfusion for severe anemia. Antiemetics, antipyretics and anticonvulsants are also frequently prescribed.

The therapy guidelines are directed by severity of symptoms and the presence of complications. Uncomplicated *P. falciparum* malaria is treated by oral administration of one of the ACTs mentioned above, typically for three days. In contrast, first line therapy of severe malaria includes parenteral (intramuscular or intravenous) artesunate. (34, 35). If parenteral artesunate is not available, artemether or quinine can be administered (34). As soon as the general condition of the patient allows, therapy is completed by a complete course of an ACT. In practice, severe malaria is defined by various severe symptoms or laboratory findings as explained in *1.1.1 The disease*. A set of clinical scores and guidelines aid in the decision which level of clinical care, as for example blood transfusion, is required (34-36).

Uncomplicated *P. vivax, P. ovale, P. knowlesi* and *P. malariae* infections can be treated with either ACT or with chloroquine in areas where parasites are sensitive to chloroquine. Regarding the first two, therapy may be supplemented with

primaquine for two weeks to prevent relapse due to the hypnozoites of *P. vivax* and *P. ovale (34)*.

Apart from ACTs, some antibiotics are recommended for prophylaxis and treatment of malaria in a few cases. Doxycycline is used as prophylaxis for travellers (37, 38), as well as sulfadoxine-pyrimethamine in the form of intermittent preventive treatment for high-risk groups in endemic regions (34). Furthermore clindamycin combined with quinine is recommended for treatment of pregnant women during the first trimester (34). In case of ACT failure or unavailability, doxycycline and clindamycin are in clinical use for therapy in combination with quinine sulphate or artesunate (34, 38).

1.2 Antibiotics

The word antibiotic originates from the Greek ἀντι *anti* (against) + βίος *bios* (life), thus, antibiotics were originally understood as substances of natural sources that inhibit or kill microorganisms. However, in common usage, the term antibiotics usually refers to medicinal substances for the treatment of bacterial infectious diseases. For this reason, medical language differentiates further and defines antiinfectives or antimicrobial therapy as the heading for substances against microorganisms composed by specific subgroups consisting of antibiotics (against bacteria), antiprotozoals (against protozoa), antifungals (against fungi), antivirals (against viruses) and anthelmintics (against worms) (39). However, many drugs act against different microorganisms and can thus be assigned to different subgroups (40, 41).

Since antibiotics are primarily developed against bacterial infections, their mechanism of action often involves processes that are exclusively present in prokaryotes. In the protozoan *Plasmodium*, there are two prokaryotic structures that were incorporated by endosymbiosis: the mitochondrion and the apicoplast (42, 43). These should be considered in more detail when analysing the mechanism of action of antibiotics against *Plasmodium* species.

In the following chapters, different groups of antibiotics are presented and their implementation or research against *Plasmodium* spp. is highlighted.

1.2.1 Tetracyclines

The first tetracycline was isolated in the late 1940s as a product of *Streptomyces aureofaciens* and *Streptomyces rimosus.* Currently, this class of antibiotics are divided into four groups according to their date of origin: natural first-generation (1948-1963: chlortetracycline, oxytetracycline, tetracycline, demeclocycline), semisynthetic second-generation (1965-1972: e.g. minocycline, doxycycline, lymecycline, meclocycline, methacycline, rolitetracycline), glycylcyclines (tigecycline) and new tetracyclines (eravacycline, sarecycline, omadacycline) (44). Over the past century, hundreds of different tetracycline derivates have been synthesized and evaluated (45, 46). As their name suggests, they have a common linear fused four ring structure and differ in the presence or absence of functional groups, which defines their pharmacological properties.

Tetracyclines belong to the broad spectrum antibiotics as they are effective against numerous pathogens, including a wide range of gram positive and negative bacteria, intracellular bacteria from the genus *Chlamydiae*, *Mycoplasmae* and *Rickettsiae*, atypical organisms such as *Treponemae, Bartonellae* and *Borreliae* and protozoans such as *Balantidium coli, Entamoeba* species and *P. falciparum (47)*.

In bacteria, tetracyclines bind to the 30S subunit of ribosomes and thus modify the attachment of aminoacyl-tRNA. During the elongation process the aminoacyl-tRNA cannot align itself correctly on the side of the 50S subunit to carry out a peptidyltransferase reaction. This results in the termination of the peptide chain during protein synthesis (46).

Most tetracyclines proved to be slowly active against *P. falciparum in vitro* including minocycline (IC50 6 days 3D7: 264 nM), tetracycline (IC50 6 days 3D7: 360 nM), doxycycline (IC50 6 days 3D7: 354 nM) and eravacycline. All of them exhibited the mentioned delayed death effect and, except for minocycline which has not yet been tested, target the apicoplast (31, 48, 49) (Figure 2). Eravacycline should be highlighted here, as it showed high activity with an IC50 of 14 nM after six days in a culture adapted *P. falciparum* clone (3D7), well below other tetracyclines. Curiously, the delayed death phenomenon of eravacycline could

not be confirmed in *P. falciparum* clinical isolates (49). Concerning *P. knowlesi*, only a few tetracyclines have been tested so far. Doxycycline showed a slightly lower delayed death effect in *P. knowlesi* compared to *P. falciparum*, with an IC50 after three cycles of 2061 nM (50).

Tetracyclines such as minocycline, doxycycline, and tetracycline clear *P. falciparum* in vivo but act slowly, requiring ≥5 days of treatment and showing delayed symptom resolution. Oxytetracycline and chlortetracycline were ineffective (51-53). In contrast, treatment with oxytetracycline or chlortetracycline yielded no parasite clearance (54, 55). Novel 7-position modified tetracyclines demonstrated potent in vitro activity (IC_{50} < 30 nM at 96 h) and cleared *P. berghei* in mice after 7 days at 10 mg/kg intraperitoneally, outperforming oral doxycycline (56).

Tigecycline, a glycylcycline developed to overcome bacterial resistance, showed variable in vitro activity against *Plasmodium* spp. (57-59). On the one hand, tigecycline is described as the only fast-acting tetracycline with IC50 values about 500-700 nM after three days in clinical isolates of *P. falciparum* (60, 61). In contrast to these findings, *Held et al.* previously stated a delayed death effect of tigecycline as IC50s after three days (2 µM) were 10-fold higher than IC50s after six days (200 nM) in culture adapted strains and clinical isolates from Gabon (48). Tigecycline was also tested *in vivo* in mice infected with *P. berghei* and was able to successfully suppress parasitaemia with a 3.7 mg/kg dose administered once daily for four days but not to cure the mice. In combination with a subcurative dose of chloroquine (33.3 mg/kg), tigecycline (33.3 mg/kg) potentiated the effect of chloroquine and was fully efficacious, an effect also described in vitro for chloroquine resistant strains (62).

1.2.2 Macrolides

Macrolides are characterized by a 12- to 18-membered lactone ring with glycosidically bound sugars and include natural and semisynthetic substances (63). Currently, only azithromycin, clarithromycin, erythromycin and fidaxomicin are FDA-approved macrolides, although several potent derivatives are available (64-67). Structural modifications of erythromycin led to ketolides and

fluoroketolides, designed to counteract resistance by binding multiple sites on the bacterial ribosome—two for telithromycin and cethromycin, three for solithromycin (68). Telithromycin, the only FDA approved ketolide (withdrawn in the EU) is indicated for community-acquired pneumonia (CAP), acute exacerbations of chronic bronchitis (AECB), and acute maxillary sinusitis (AMS) in adults (69, 70).

Macrolides act bacteriostatically by binding the 50S ribosomal subunit, blocking translocation during protein elongation (71, 72). Their antimicrobial spectrum includes intracellular pathogens such as *Chlamydiae, Mycoplasmae, Rickettsiae, Legionellae* and various gram positive and negative bacteria (64). Typical side effects are mild (mostly gastrointestinal), while severe events like hepatotoxicity or QT prolongation are rare (73). It should be noticed that macrolides inhibit CYP3A4 leading to drug interactions (74). Safety during pregnancy is only established for erythromycin (75). Advantages include diverse formulations, e.g. oral, injectable, and topical (76).

In malaria, macrolides can present a slow or fast onset of action. The slow ones include erythromycin, azithromycin and clarithromycin which exhibit activity with IC50s after 5 days of 5800 nM, 60 nM, and 6400 nM respectively by targeting the apicoplast (31) (Figure 2). Azithromycin was also evaluated *in vitro* against *P. knowlesi* and exhibited an IC50 of 31.9 nM after 81 hours (50).

In contrast, fast-acting macrolides such as borrelidin, kitasamycin (synonym: leucomycin) and avermectins, exhibit IC50s in nanomolar range within the first cycle (31, 77, 78). *Otoguro et al*. were the first researchers describing borrelidin´s *in vitro* activity (IC50 against K1: 1.9 nM, IC50 against FCR3: 1.8 nM after three days) against *P. falciparum* and *in vivo* efficacy against *P. berghei* and *P. yoelii* spp. (77). Borrelidin exhibited a higher activity against *P. yoelii* (ED50 0.07 mg/kg) and *P. berghei* (ED50 0.18 mg/kg) in malaria infected mice than the reference drugs artemether (ED50 1.1 mg/kg and 0.95 mg/kg), artesunate (ED50 0.4 mg/kg and 1.7 mg/kg) and chloroquine (ED50 4.5 mg/kg and 1.5 mg/kg). Toxicity data are conflicting: some studies report acceptable *in vitro* profiles, others deem it too toxic (77, 79-81). To develop this promising drug in malaria research, *Novoa et*

al. synthesized less toxic borrelidin analogues with retained efficacy (82). Despite these achievements in 2014, no clinical data is available for borrelidin.

Regarding leucomycin (3D7: IC50 ~ 50 nM), activity was also shown *in vivo* in *P. yoelii* infected mice experiments by supressing parasite growth by 78 % at daily administration of 50 mg/kg for three days (78).

Regarding avermectins, the most studied semisynthetic derivative ivermectin improved nutritional situation through its use in food production and contributed significantly to the health status of billions of people in the (sub-)tropics due to its broad-spectrum activity to endo- and ectoparasites especially threatening the poorest of our world (83, 84). It is approved for rosacea (85), gastrointestinal strongyloidiasis, lymphatic filariasis, scabies, onchocerciasis, head lice and trichuriasis (86-88). It acts via paralysis-inducing chloride channel modulation in parasites (84).

Apart from its success in treatment nematode and arthropod infections, ivermectin has gained attention for malaria control. One strategy targets transmission by reducing mosquito survival and disrupting sporogony (89-92)); the other focuses on its *in vitro* activity against hepatic and blood-stage parasites (90). Ivermectin inhibited the *in vitro* parasite growth during asexual (IC50 3D7 three days: 100 nM) and sexual (IC50 559 nM) blood stages (90). It also impaired liver-stage development *in vitro* in human hepatoma cells and *in vivo* in a mouse model (93).

Several mass drug administration (MDA) trials with ivermectin reported decreased mosquito survival and sporozoite rates (89, 91, 92, 94). Concerning the impact of MDA on incidence and prevalence of parasitemia literature reveals contradictory findings. While a 2015 cluster-randomised trial in Burkina Faso and a modelling study indicated reduced malaria incidence in children (95-97), a 2020 descriptive prevalence study observed no change, likely due to subtherapeutic drug levels (98-100).

Together, these findings support ivermectin's potential as a multipurpose tool in malaria control strategies.

In our experiments, we also evaluated the macrolide boromycin, produced by *Streptomyces antibioticus* and first described in 1967 as the first naturally occurring boron-containing compound (101-103). With regard to its range of effectiveness, boromycin shows activity against Gram-positive bacteria (104), *Mycobacteria (104)*, *HIV* (105) and protozoan parasites like *Babesia* (103), *Toxoplasma gondii* (106) and *Cryptosporidium parvum (106)*. Concerning malaria, its high antiplasmodial activity is already published once *in vivo* in *P. berghei* infected mice (ED50: 2.2 mg/kg) and in *P. gallinaceum* infected birds (ED50: ~ 7 mg/kg) (107). The mode of action of boromycin was investigated in *Bacillus subtilis* and later confirmed in *Mycobacterium bovis (102, 104)*. It is stated, that boromycin is an ionophore for potassium ions, examined by rapid loss of membrane potential, reduction of intracellular ATP and leakage of cytoplasmatic proteins. Importantly, addition of 20 mg/mL KCl reversed its effects, reducing activity from 50% to 10% at IC50 levels (104), confirming its ionophoric mode of action.

1.2.3 Folate synthesis inhibitors and Antifolates

Folate synthesis inhibitors, as sulphonamides, competitively inhibit (108), whilst antifolates, as proguanil, pyrimethamine and trimethoprim (109), antagonise the effect of vitamin B9 (folic acid) mostly by inhibiting dihydrofolate reductase (DHFR) (110). For treatment of infectious diseases antifolates are often combined with sulphonamides, with sulfadoxine-pyrimethamine (SP) and cotrimoxazole (trimethoprim and sulfamethoxazole) being the most used combinations. SP belongs to the first-line medication for chemoprevention of malaria in pregnant women and children (111). In infants, a distinction is made between perennial malaria chemoprevention, which consists of three doses of only SP in regions with year-round malaria, and seasonal malaria chemoprevention, which is monthly administration of antimalarial medicines, usually SP plus amodiaquine, for as long as the rainy season lasts (111).

Cotrimoxazole widely used for infections such as urinary tract infections, *Pneumocystis jirovecii* pneumonia and shigellosis (112), is recommended for daily prophylaxis in HIV patients to prevent bacterial, fungal and plasmodial

infections (113). Its use in malaria-endemic regions has reduced incidence and mortality in children (114-118). Although concerns exist regarding cross-resistance with sulfadoxine-pyrimethamine (SP), cotrimoxazole remains effective against SP-resistant *P. falciparum*, with no evidence of selecting for SP resistance mutations in DHFR or DHPS (119, 120).

1.2.4 Fosmidomycin

Fosmidomycin, produced by *Streptomyces lavendulae*, hinders the first step of the MEP pathway by inhibiting 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DOXP) (121). This disruption is lethal to organisms reliant on this pathway, including multidrug-resistant Gram-positive and Gram-negative bacteria, plastid-containing eukaryotes, and plants (122).

In *P. falciparum*, fosmidomycin and its analogue FR900098 have a fast onset of action, inhibiting parasite growth after one cycle (IC50 ~ 1 µM). Growth rescue via isopentenyl pyrophosphate (IPP) confirms the apicoplast as the target (31, 123) (Figure 2). *In vivo* efficacy was proven in rodent *P. vinckei*-infected mice cured after fosmidomycin administration every eight hours for four days (123). A clinical trial in Gabon and Thailand showed initial parasite clearance with 1200 mg oral fosmidomycin every 8 hours for 7 days, but recurrence occurred in 22% (Gabon) and 78% (Thailand) by day 28 (124). To avoid recurrence, a combination with a slow-acting drug like clindamycin, which showed synergistic activity *in vitro* as well as in a mouse model, was proposed (125). In clinical trials, the lowest dose regimen (900 mg fosmidomycin and 300 - 600 mg clindamycin every six hours for at least five days) resulted in an approximately 90 % 28 day cure rate (126) In contrast, four years later the same regimen in similar doses (30 mg/kg fosmidomycin and 10 mg/kg clindamycin) in children under three years showed high efficacy after 7 days (94.6 %) but many recrudescence after 28 days with an cure rate of only 40.5 % (127). Furthermore, the combination artesunate (1 to 2 mg/kg of body weight) and fosmidomycin (30 mg/kg of body weight) achieved 100 % cure on day 28 with a three-day regimen in children (128). In the latest clinical trial, the combination fosmidomycin (30 mg/kg twice daily) with piperaquine (16 mg/kg once daily) for three days achieved a 100 % cure on day

28 (129). Despite the promising results, fosmidomycin is not yet approved for malaria therapy (130).

1.2.5 Lincosamides

Lincosamides are a group of antibiotics derived from the natural products lincomycin and celesticetin, which are produced by different *Streptomycin* species. Lincosamides include mirincamycin, pirlimycin and clindamycin, whereby only the latter is approved for human use. Clindamycin is especially used in soft tissue infections with *Staphylococcus*, *Streptococcus* and *Anaerobes* due to its high bioavailability and its capability to distribute into fluids and tissues (131-133). Lincosamide antibiotics interfere with protein biosynthesis by binding to the 23S part of the 50S subunit of the prokaryotic ribosome, blocking the peptidyl transferase centre and causing dissociation of the tRNA from the ribozyme and amino acid chain termination and thus have a bacteriostatic effect (134). *In Plasmodium* spp. clindamycin also inhibits the prokaryotic protein biosynthesis in the apicoplast (31) (Figure 2).

Clindamycin is already an established FDA approved antimalarial in combination with quinine to treat pregnant women in the first trimester or in combination with quinine or artesunate for the oral follow-on treatment of severe malaria if ACT is not available (135). In addition, *in vitro* studies showed that mirincamycin´s cis and trans isomers have higher activity after six days against *P. falciparum* isolates (IC50 3.2 nM and 2.6 nM) when compared to the reference drug clindamycin (IC50 12 nM) (136). Regarding *P. knowlesi*, clindamycin and mirincamycin exhibited similar IC50s to *P. falciparum* against *P. knowlesi* with IC50s after multiple cycles of 15.9 nM and 2.5 nM respectively (50, 137). Moreover, clindamycin was tested *in vivo* in *P. knowlesi* infected rhesus monkeys, where it cured infection after daily administration for five days with a slower onset of action than chloroquine (138).

1.2.6 Aminoglycosides

Aminoglycosides, first isolated in 1944, are a heterogeneous group consisting of large molecules related to carbohydrates. They bind to the 30S subunit of ribosomes and thus prevent protein biosynthesis (139). To exert this effect,

however, they must first reach their target, but since they cannot pass through eukaryotic cell membranes due to their size and hydrophilicity, they are ineffective against intracellular pathogens. This leads to their ineffectiveness against intracellular *Plasmodium* spp., that is why aminoglycosides are used to prevent the growth of other microorganisms in *in vitro Plasmodium* cultures (140). Aminoglycosides are mainly used for infections with problematic pathogens, especially gram negative enterobacteria (141).

1.2.7 Nitroimidazoles

Nitroimidazoles, with the most important derivatives metronidazole, tinidazole and nimorazole, unlike most of the antibiotics discussed so far, do not act on the ribosome but directly on bacterial nucleic acids. More precisely, nitroimidazoles cause DNA damage by binding to the base pairs, which in turn can lead to strand breaks. This is only possible due to their chemical structure, which consists of an imidazole substituted with a nitro group. This nitro group can be reduced by anaerobic metabolism and thus becomes an active metabolite that can bind the base pairs (142, 143). The spectrum of activity of this bactericidal class includes anaerobic bacteria and protozoa (144).

Literature on antimalarial activity of nitroimidazoles offers contradictory findings as, first, in 1984 clearance of parasitaemia of patients infected with *P. vivax* after metronidazole treatment was observed (145). However, a second study also assessed the effect of metronidazole on malaria parasites in children with coinfection of *Giardia lamblia* and *P. falciparum*. In contrast to the previous study, this time only a reduction in malaria parasites could be achieved, but no clearance (146). An *in vitro* study of several antibiotics in 2001 considers metronidazole to be inactive (147).

1.3 Objectives of this study

Malaria treatment strategies are continuously evolving as *Plasmodium spp.* populations adapt to first-line therapy through to the development of resistance. One of the major challenges in malaria control in the 20th century was the emergence of chloroquine resistant parasites (148). Chloroquine was the gold standard treatment at that time and so the need for other medicines to treat chloroquine resistant parasites and prevent the spread of these strains was urgent. Hence, SP and then ACT have replaced chloroquine as first-line therapy. Of particular concern was therefore the identification of artemisinin resistant parasites in Cambodia in 2008 (149)(149). Since then, artemisinin resistance has been reported across the Greater Mekong Subregion, as well as in Guyana, in Papua New Guinea and recently also in Uganda and Rwanda (150-154). Unlike chloroquine resistance, artemisinin resistance is mainly referred to as partial resistance to highlight its time-limited and cycle-specific feature. It primarily affects the ring stage of the parasite life cycle and is associated with delayed parasite clearance (defined as a parasite clearance half-life > 5 hours), although it does not generally lead to complete treatment failure (155-158). Complete treatment failure is only observed when resistance to the ACT partner drug is also present (157). Despite ACT failures in some regions, the WHO continues to recommend ACT therapy, advising a switch to one of the six approved ACTs as first-line treatment where resistance occurs. The pharmacodynamic interplay between the fast-acting artemisinin derivative and the longer-acting partner drug creates a self-reinforcing resistance dynamic: reduced artemisinin efficacy results in prolonged parasite survival, which increases the burden on the partner drug. This elevated exposure can select for resistance to the partner compound, undermining treatment efficacy and further promoting artemisinin resistance (154, 156, 158).

As explained by Nguyen et al in 2023 a new approach is the introduction of triple therapy with two partner drugs to prevent resistance extension (159).

The aims of this study are:

- to screen *in vitro* activity against *P. falciparum* and *P. knowlesi* of antibiotics by drug sensitivity assays.
- to characterize antiplasmodial activity of the tested drugs by
    - evaluating first cycle or second cycle activity.
    - validating the apicoplast as a drug target of the most active antibiotics and the ones presenting a delayed death effect.
    - exploring ionophoric activity of the most active antibiotic.
    - analysing stage specificity of the most active antibiotic.
- to determine cytotoxicity on HEPG2 cells of the most active antibiotic.
- to establish an inhouse standard operating procedure for drug sensitivity assays for A1H1 *P. knowlesi*.
- to culture *P. knowlesi* A1H1 *in vitro* without supplementation of human serum to standardize drug sensitivity assays and reduce costs in continuous culture.
- to compare drug susceptibility against the tested antibiotics of different species (*P. falciparum* and *P. knowlesi*) and strains (3D7 and Dd2).

2 Materials und methods

2.1 *Plasmodium falciparum* and *Plasmodium knowlesi* culture *in vitro*

P. falciparum laboratory strains 3D7 (chloroquine sensitive, provided by BEI resources, MRA-102) and Dd2 (chloroquine, sulfadoxine and pyrimethamine resistant, quinine tolerant (160, 161), provided by BEI resources, MRA-150) were cultivated *in vitro* as described before (162). For this purpose*, P. falciparum* were grown in human 0 Rh+ erythrocytes obtained from the Blood Bank of the University of Tübingen (at 2.5 % haematocrit) and complete medium consisting of RPMI-1640 Medium (Sigma-Aldrich, R0883) supplemented with 1 M HEPES solution (2.4 % v/v) (Sigma-Aldrich, ref: H0887), 200 mM L-glutamine (1.2 %) (Gibco, ref: 25030-024), 50 µg/ml gentamicin (0.0001 % v/v) (gibco, ref: 11520506) and AlbuMax II stock solution (10 %). One Litre AlbuMax II stock solution contained 10.4 g RPMI-1640 (gibco, Ref: 51800-035), 5.96 g HEPES (Sigma-Aldrich, Ref: H4034-1KG), 3,34 g $NaHCO_3$ (Sigma-Aldrich, Ref: S5761-500G), 2 g D-Glucose (Sigma-Aldrich, Ref: G8270), 0.2 g hypoxanthine (Sigma-Aldrich, Ref: H9377) and 50 g AlbuMax II Lipid-Rich BSA (gibco, Ref: 11021-037).

P. knowlesi laboratory strain A1H1 (kindly provided by Robert Moon of LSHTM, London, UK under an MTA by the Francis Crick Institute) was cultivated as described for *P. falciparum* with some modifications (163). Initially, the parasites were cultivated in complete medium as described before, with addition of 5 % human AB serum. To establish *P. knowlesi* culture in our laboratory, the serum was replaced by 20 % v/v of Albumax II solution once the parasite multiplication became constant (one month after thawing) and furthermore (two weeks later) by 10 % v/v of Albumax II solution and cultivated for four months.

P. falciparum and *P. knowlesi* cultures were maintained at 5 % CO_2, 5 % O_2, at 37°C in the Thermo Scientific Heracell 500i CO_2 Incubator. The medium was changed every two to three days and parasite culture was diluted with fresh erythrocytes, if necessary, to maintain a parasitaemia below 5 %.

2.2 Quantification and dilution of parasites

A thin blood smear was prepared and stained with 5 % Giemsa solution (Giemsa´s azur eosin methylene blue solution for microscopy, Merck, ref: 109204) in phosphate buffer (sodium phosphate dibasic heptahydrate (MW: 268.07g/mol) and sodium phosphate monobasic monohydrate (MW: 137.99 g/mol), 0.1 M, pH 7.2) to quantify the parasitaemia and to identify the parasite stages. A blood drop was spread out on a slide and dried in the sterile bank. Afterwards, the slide was fixed in > 99.8 % methanol (Honeywell Riedel-de Haen, ref: 15614740) for 10 seconds and stained in 5 % Giemsa solution for at least 15 minutes. The slide was washed with water, dried, and then analysed with a 100x immersion oil objective lens under the microscope. The number of parasites in 1,000 red blood cells were counted and the percentage of infected cells was calculated to decide whether dilution was necessary to obtain a parasitaemia below 5 %.

2.3 Synchronization of parasites

P. falciparum parasites were synchronized before the drug sensitivity assays with magnetic column separation with LS columns (ref: 130-042-401, Miltenyi Biotec GmbH) in Macs Multi Stand (ref: 130-042-303, Miltenyi Biotec GmbH) two days before and on the day of the drug or stage specific assay (164).

P. knowlesi (parasitaemia around 5 %, cultured in 5 % human serum) was synchronized with Nycodenz density gradient to recover mature schizonts as described before (50, 163, 165). For this purpose, the culture was centrifuged, the supernatant removed, and 10 ml of medium were added to the cell pellet. Then, the dissolved pellet was layered over 5 ml NycoPrepTM 1.077 (14.1 % Nycodenz, 0.44 % of 5mM NaCl Tricine/NaOH, pH 7.2, Produced by Fresenius Kabi Norge AS for Axis-Shield, ref: 1114550) in a 15 ml Falcon tube covered in aluminium paper as NycoPrep is light sensitive. After centrifugation at 800 x g for 20 minutes with low brake/acceleration, cells were collected separately. The isolated schizont interface was removed, washed, and resuspended in medium supplemented with 5 % serum, filled up to 1.5 % haematocrit and cultured at 5 % CO_2, 5 % O_2, at 37°C in the Thermo Scientific Heracell 500i CO_2 Incubator. After

approximately six hours, the schizonts had developed to early ring stage that were used for starting the drug sensitivity assays. Before performing drug sensitivity assays, the medium supplemented with 5 % human serum was replaced by 20 % v/v of Albumax II medium to not interfere with the tested drugs (166).

2.4 Drug solutions

Tested drugs were dissolved in dimethyl sulfoxide (DMSO), ethanol or distilled water, according to supplier recommendations, to prepare 50 mM or 100 mM stock solutions depending on solubility. A list of the tested drugs, the supplier, and the used solvent to prepare the stock solution can be found in Table 1. Afterwards, the stock solutions were diluted to a working solution with complete RPMI-1640 medium to the maximum concentration tested to avoid any toxic effect of the solvent *(*Table 1). First, we started with a working solution of 1 mM based on data of already tested antibiotics (Table 1) and subsequently adjusted to ensure IC_{50} values fell within the optimal dynamic range, including no and full inhibition to covert he full dose-response-curve. Final solvent concentration in the drug sensitivity assays was 1 %, thus lower than the concentration of DMSO and ethanol that interfere with parasite growth as shown previously (167). Furthermore, DMSO has been used as a solvent for drug-assays to assess more than 100 compounds at our group. As toxicity against P. falciparum and P. knowlesi parasites was never observed at relevant concentrations, an additional DMSO-only control was not included. Doxycycline, chloroquine, quinine, ivermectin and chlorotonil A were defined as control drugs for the three-day assays (31, 48, 90, 168, 169). Clindamycin hydrochloride was used as a reference drug for the six-day assays (see *2.5 Drug sensitivity assays for ring stage P. falciparum and P. knowlesi* (31, 48, 170).

Antibiotics were selected based on the following criteria: lack of prior testing against *P. falciparum* or *P. knowlesi*, existing clinical approval or development status, and market availability. The final selection focused on diverse antibiotic classes. Tetracyclines and macrolides were included based on reported antiparasitic activity, while nitroimidazoles and aminoglycosides were selected based on laboratory availability and absence of existing activity data.

Table 1 Antibiotics tested against P. falciparum and P. knowlesi: This table lists the antibiotics tested in drug assays against P. falciparum and P. knowlesi, along with their respective suppliers, molecular weights, solvents used for dilution, working concentrations employed in the assays, and relevant references. The suppliers are indicated as follows: SA – Sigma-Aldrich, CC – Cayman Chemicals, SCBT – Santa Cruz Biotechnology, Gibco – Gibco by Life Technologies, and HZI – Helmholtz Centre for Infection Research

(31)Antibiotic class	**Name of antibiotic**	**Solvent**	**Company**	**MW in g/mol**	**Working solution 3 and 6 days**	**Related References**
Tetracyclines	Minocycline hydrochloride	water	SA	457.48	1 mM	(48, 147, 171, 172)
	Chlortetracycline	DMSO	CC	478.9	500 µM	(54, 55, 173)
	Demeclocycline	water	SCBT	464.85	1 mM	(171, 172, 174, 175)
	Lymecycline	water	SCBT	602.63	500 µM	(172)
	Meclocycline	DMSO	CC	476.9	1 mM	-
	Methacycline	DMSO	SCBT	442.42	1 mM	-
	Sarecycline HCl or P005672 HCl	DMSO	Adooq Bioscience	524	1 mM	-
	Omadacycline	DMSO	Hycultec	593.11	1 mM	-
	Eravacycline	DMSO	Hycultec	558.56	1 mM	(49)
Macrolides	Borrelidin	DMSO	SCBT	489.64	20 µM/ 4 µM	(31, 77)
	Boromycin	DMSO	SCBT	879.87	250 µM	(107, 176)
	Josamycin	Ethanol	SCBT	827.99	1 mM	(172)
	Oleandomycin	DMSO	SCBT	687.86	1 mM	-
	Troleandomycin	DMSO	SCBT	813.97	1 mM	(172)
Nitroimidazoles	Tinidazole	DMSO	CC	247.3	1 mM	(175)
	Nimorazole	DMSO	SA	226.23	1 mM	-
	Metronidazole	DMSO	SA	171.17	1 mM	(145-147)
Aminoglycosides	Kanamycin sulfate	water	SA	484.5	1 mM	(140)
	Streptomycin sulfate	water	SA	581.57	1 mM	(140)
	Gentamicin	water	Gibco	477.596	1 mM	(140)
Controls 3 days	Chloroquine	water	SA	515.86	50 µM	(31, 50, 168)
	Quinine	DMSO	SA	324.42	50 µM	(50, 168)
	Ivermectin	DMSO	SA	877.1	250 µM	(89-93, 175)
	Doxycycline hyclate	water	SA	512.94	1 mM/ 500 µM	(31, 48, 50, 147, 169)
	Chlorotonil A	DMSO	HZI	479.44	20 µM	(48, 177)
Control 6 days	Clindamycin HCl	water	SCBT	461.44	1 mM / 200 µM	(31, 48, 137, 170)

2.5 Drug sensitivity assays for ring stage *Plasmodium falciparum* and *Plasmodium knowlesi*

To determine the activity of the drugs in Table 1 against *P. falciparum* and *P. knowlesi*, we carried out drug sensitivity assays according to standard procedures (178). First, serial three-fold dilutions (eleven concentrations and one drug-free negative control column) of the drugs were distributed in duplicate into 96-well tissue culture plates (FALCON 353075; Corning Inc.). Afterwards, the synchronization of parasite cultures was determined by microscopy with a cut-off of > 90 % ring stage as described above in section *Quantification and dilution of parasites* and *Synchronization of parasites*. The parasitaemia of *P. falciparum* and *P. knowlesi* was adjusted to 0.05 % (if measured with HRP2 ELISA, see *2.6 Histidine-rich protein 2 detection for P. falciparum*) or 0.5 % (if measured with Sybr Green-I assay, see *2.7 Sybr Green-I detection for P. knowlesi*) and the parasites were seeded at a haematocrit of 1.5 % (HRP2 ELISA) or 2 % (Sybr Green-I assays) in a total volume of 225 µl and incubated with 5 % CO_2 and O_2 at 37°C for 72 (three-days assay) and 144 hours (six-days assay). The table below (Table 2) illustrates an overview of the different drug sensitivity assay conditions.

Table 2 Overview of different experiment conditions of the drug sensitivity assays: This table summarizes the experimental conditions applied in drug sensitivity assays, categorized by the measurement method (HRP2 ELISA or Sybr Green-I) and the parasite species tested. It includes haematocrit and parasitaemia levels for each method and species.

Measurement method	**Haematocrit in drug sensitivity assay**	**Parasitaemia in drug sensitivity assay**	**Parasite species tested**
HRP2 ELISA	1.5 %	0.05 %	*P. falciparum*
Sybr Green-I	2 %	0.5 %	*P. knowlesi*

Drug sensitivity of *P. falciparum* was measured by HRP2 ELISA (after plates were frozen and thawed three times) to quantify the antigen histidine-rich protein 2 (HRP2). In contrast do 3D7, Dd2 has a deletion of the gene coding for HRP2 but possesses the antigen HRP3. The antibodies of our HRP2-ELISA cross-react with HRP3. As there is no HRP2-paralouge in *P. knowlesi* that is bound by the

anti-HRP2 antibodies of the ELISA, drug susceptibility was only analysed by Sybr Green-I assay, that binds to DNA and builds a complex with the parasitic DNA (179)

Every drug was tested in duplicate and repeated at least twice with HRP2 ELISA and Sybr Green-I assay.

2.6 Histidine-rich protein 2 ELISA for *Plasmodium falciparum*

The HRP2 ELISA was performed as described by *Noedl et al.* (180). 96-well enzyme immunoassay (EIA)/ radioimmunoassay (RIA) plates with Flat Bottom without Lid, High Binding (Costar 3590; Corning Inc.) were precoated with the first antibody MPFM-55A (Purified Mouse anti-*P. falciparum* HRP2, icllab (Port-Island, OR, USA), diluted to 1 µg/ml in phosphate buffered saline (PBS) (Gibco, Ref: 11510546), overnight. After discarding the content, blocking with 2 % bovine serum albumin in PBS (SERVA, ref: 11920) and washing with PBS/Tween 20 (0.05 %) (Sigma, ref: P9416), samples from the drug assays (50 µl of 3D7 and 100 µl of Dd2) were transferred to the precoated plates. However, in the assays with 3D7 only 50 µl of sample and 50 µl of ddH_2O were transferred from the culture plate to the ELISA plate as the strain 3D7 has HRP2. As Dd2 only possesses the cross-reacting HRP3, in these assays 100 µl sample were transferred.

Following one hour incubation time, plates were washed with PBS/Tween 20 (0.05 %) again and the diluent with the second antibody MPFG-55P (Anti-*P. falciparum* HRP2 Peroxidase Conjugated - Mice IgG (Monoclonal), icllab (Port-land, OR, USA)) was added (PBS with 2 % BSA, 1 % Tween 20 and 0.05 µg/ml of the second antibody). The plates were incubated for one hour and subsequently washed. Finally, 100 µl of substrate TMB chromogen (ECO-TEK, Kem-EN-Tec-Diagnostics, ref: 4380/4430) was added per well, and the reaction was stopped after five to seven minutes with 50 µl 1 M sulphuric acid (Carl Roth GmbH, ref: X945.1). The Optical Density (OD) per drug concentration was measured with a microplate reader (CLARIOstar, BMG Labtech) at the excitation filter of 450 nm.

2.7 Sybr Green-I detection for *Plasmodium knowlesi*

The Sybr Green-I detection was carried out as detailed on p. 122 of *Methods in malaria research, 2013 (181)*. First, 0.4 µl Sybr Green-I (ThermoFisher, ref: S7563) was applied per ml 1x Lysis Buffer containing 10 mM Tris, 2.5 mM EDTA, 0.004 % wt/vol saponin, 0.04 % vol/vol Triton X-100 filled up to one litre with ddH_20. Afterwards, 30 µl of this mixture were added to each well of a 96-well half area, non-transparent white microplates (Greiner 675075, Bio-one) with a multichannel pipette. Subsequently, 30 µl/well of the thawed culture plate were transferred to the half area microplate. The whole last column was a drug-free negative control. After one hour incubation at room temperature in the dark, fluorescence was measured using a microplate reader (CLARIOstar, BMG Labtech) at 490 nm excitation and 540 nm emission wavelength.

2.8 Characterization of the antiplasmodial activity

2.8.1 IPP supplementation assay

Drug sensitivity assays were repeated with supplementation of 200 µM isopentenyl pyrophosphate trilithium salt (IPP) (Sigma, ref: 00297) in the culture medium to identify apicoplast-targeting inhibitors, as described by *Yeh and DeRisi* (25). They show that if parasites continue to grow after supplementation with IPP, the apicoplast is confirmed as the target, as asexual parasites without apicoplast can only survive when supplemented with IPP. However, if supplementation with IPP does not rescue the parasites from the drug's effect, *it* his highly unlikely that the apicoplast is the primary target.

Drug sensitivity assays were initiated at ring stage at the same conditions as described before in the section 2.5 *Drug sensitivity assay*. The IPP supplementation assay was performed with minocycline, josamycin, borrelidin and boromycin in 3D7 and eravacycline, minocycline, josamycin in A1H1; clindamycin was used as control. The selection of drugs was based on the presence of delayed death effect as seen in the activity difference between the three- and six-day assay of at least 20-fold difference (minocycline, josamycin and eravacycline); in addition, the drugs with the highest activity at low nanomolar

concentrations were tested (borrelidin and boromycin). For control purposes, all plates were also prepared without IPP supplementation at the same time.

After 144 hours of incubation, 3D7 assays were measured with HRP2 ELISA and A1H1 assays were evaluated with Sybr Green-I detection to assess whether parasite growth has been rescued by the addition of IPP. Again, IPP supplementation was tested in duplicate and repeated at least twice with HRP2 ELISA or Sybr Green-I assay.

2.8.2 KCl supplementation assay

Further on, we examined the most active substance boromycin more closely for its mode of action. In the literature, potassium channels have been described as the molecular target of boromycin in *Bacillus subtilis* and *Mycobacterium tuberculosis*. Therefore, we also investigated the role of potassium flow in *P. falciparum (104)*. First, we established 220 µg/mL of KCL (Sigma, ref: 1049360250) and $MgCl_2$ (Sigma, ref: M4880) as non-toxic concentrations by a drug sensitivity assay as explained in previous chapter. Based on the experiments of *Moreira et al*, we performed a drug sensitivity assay with two-fold dilution of boromycin or the control drug chloroquine with the addition of either 220 µg/mL KCL or 220 µg/mL $MgCl_2$ to all wells to determine a possible inhibition of the activity of boromycin by providing excess KCl in the medium. If the potassium channels are also the target in *P. falciparum*, the addition of KCl would increase the IC50 while it should not be altered by the negative control $MgCl_2$. As in the other experiments, KCl and $MgCl_2$ experiments were tested in duplicate and repeated at least twice.

2.8.3 Stage specific assay

To determine at which stage parasites are vulnerable to boromycin, we performed a stage specific assay twice independently of each other. Assessing stage specificity in antimalarial drug evaluation is crucial, as targeting early stages—particularly ring forms—provides insight into the drug's mode of action, limits parasite replication, prevents progression to more pathogenic stages, and offers a complementary mechanism well-suited for combination therapies (90). Boromycin 1 nM (approx. IC50) was incubated with MACS synchronized *P.*

falciparum strain 3D7 in ring, trophozoite or schizont stage at 1 % parasitaemia. For control purposes, the synchronized parasites were also incubated without drug. At time points 0h, 6h, 12h, 24h, 36h, and 48h a thin blood smear was prepared and the percentage of rings, trophozoites, schizonts and dead parasites were counted under a Leica DMBL microscopy and pictures were taken using ProgRes C10 camera and software (Jenoptik) at 100x magnification.

2.9 Drug cytotoxicity testing

To determine drug toxicity to a human cell line, an established neutral red cytotoxicity screening was performed on hepatocarcinoma cells (HepG2) (182). We decided to use HepG2 cells because, on the one hand, they represent better than other cells host cells for Plasmodium (i.e. during liver stage) and, on the other hand, there is an already established cytotoxicity assay for this cell line (182-184).

HepG2 cells (obtained from ATCC; HB-8065), a human hepatocyte carcinoma cell line, were grown in RPMI-1640 (Sigma Aldrich) supplemented with heat-inactivated fetal bovine serum (10 % v/v) (Sigma Aldrich), 6 ml 200 mM L-glutamine (Gibco) and 6 ml 5 mg/ml penicillin/streptomycin solution (Gibco, ref: 15140) in the incubator (37°C, 5 % CO_2, 5 % O_2), with medium change every two days. To detach cells when they reached a semi-confluence layer, they were washed twice with PBS and incubated with Trypsin-EDTA (0.25 %) in Hank´s medium (ref: T4049, Merck) for two minutes at 37°C (185). Afterwards, 10 mL of medium was added to the flask to inactivate the trypsin. Subsequently, 10 µl of cell suspension were stained with 10 µl of Trypan Blue 0.4 % (ThermoFisher, ref: 15250061) in PBS to verify viability and counted in a Neubauer chamber and the cell suspension was centrifuged at 1800 rpm for five minutes. Supernatant was removed and cells diluted with medium until reaching 300,000 cells/ml. An aliquot of 100 µl of cell suspension was dispensed into a 96 wells plate (Corning 3599 - flat bottom, ref. 3290102, Merck) and incubated for 24 h for cell growth and adherence.

The next day, boromycin (1 mM concentration in the first well) and the reference drugs chloroquine (1 mM concentration in the first well) and quinine (1 mM

concentration in the first well) were serially diluted 1:2 in supplemented RPMI-1640 in a 96-well plate (eleven concentrations and one drug-free negative control column). The medium of HepG2 cells plates prepared the day before was removed. 100 µl of drug dilution plate were respectively transferred to the cell containing plate (Corning 3599 - flat bottom, ref. 3290102, Merck) and incubated for additional 24 hours.

On the third day, medium-drug-solution was decanted from cells and 100 µl of supplemented RPMI-1640 with 1.5 % Neutral red (3.3 mg/ml) (Merck, ref. N2889) were added per well for nuclear counterstaining and incubated for three hours at appropriate culture conditions, according to manufacturer's instructions.

After washing with PBS, 100 µl/well of lysing solution (50 % methanol, 49 % ddH_2O, 1 % acetic acid) was added and shaken for 10 min. The absorption was measured with CLARIOstar BMG Labtech at 570 nm.

In order to compare the effect of boromycin on the HepG2 cells with the activity in *Plasmodium* spp.*,* we determined the selectivity index (SI) by calculating the ratio between the IC50 in the cytotoxicity assay and the IC50 against *Plasmodium* spp. ($\frac{IC50HepG2}{IC50Plasmodium\ spp.}$). The selectivity index should be maximized to ensure therapeutic efficacy and safety. For a more rigorous assessment, we defined a threshold SI whereby the compound should be at least 100-fold more selective for the parasite than for the host (183). The cytotoxicity assay was performed twice in duplicate.

2.10 Analysis

As already established for individual inhibitory concentrations (IC50) and inhibition curves, they were calculated by nonlinear regression analysis of log concentration-response curves, using the drc v0.9.0 package for bioassays of the statistics program R v.2.3.1 (186, 187).

The drc v0.9.0 package works via mathematical model fitting to certain raw data, in our case to the independent dose and the dependent effect. The resulting model describing the dose-response relationship is then checked by model quality criteria e.g. Akaike Information Criterion or Bayesian Information Criterion.

Once a suitable model has been found, results such as the half maximal inhibitory concentration (IC50) can be calculated and a visual representation can be created. The IC50 is the concentration of a drug at which 50% of the maximum possible effect is achieved. For the analysis, we particularly use the IC50s since it is the most commonly used variable in the literature, allowing better comparison with existing studies (31, 48-50, 55, 77, 107, 145-147, 172, 174, 175). Additionally, the IC50 provides the most stable estimate under our assay conditions. For our most active substances, we also calculated the IC90 using the drc package. The IC90 is useful for determining the dose required to achieve a very high level of effect, which we aim for in the elimination of the parasites. A low IC50 by itself does not strictly provide any information about the efficacy of a drug. However, it should be emphasized that all approved malaria drugs, except for quinine, show an IC50 in the nanomolar range in vitro. Therefore, the IC50 is suitable as the first variable for sorting out active substances.

IC50 values were only included in the analysis if the drug-free control well showed at least twice the optical density values as the highest drug concentration in the HRP2 ELISA and control drugs exhibited similar results to literature (31, 50, 188-190). In fact, the following IC50 values were defined:

Chloroquine 3D7 3 and 6 days < 30 nM, Dd2 3 and 6 days > 200 nM

A1H1 3 days < 40 nM

Doxycycline 3D7 and Dd2 3 days > 4 µM, A1H1 3 days < 4 µM

Clindamycin 3D7 and Dd2 3 days > 37mM, 6 days < 20 nM

A1H1 3 days < 200 nm

The presence of a delayed death effect was defined as a fold difference of the IC50s after three and six days higher than 15. There is no exact definition in the literature; we base our approach on the fold differences of drugs that have already been classified as slow-acting in the literature (31, 170).

To evaluate the significance of the drug assay with and without IPP supplementation, we applied the non-parametric Mann-Whitney U test. Given the

very small sample size (n = 4), statistical inference about the underlying data distribution is not reliable. Although a normal distribution would be expected theoretically, this assumption cannot be robustly verified with such a small number of observations. To avoid relying on distributional assumptions and to include all data points, we chose the Mann-Whitney U test, which is robust against deviations from normality and particularly well-suited for small samples. Statistical significance was defined as $p < 0.05$, and the effect size was calculated using the following formula:

$$effect\ size\ (r) = \left| \frac{z}{\sqrt{sample\ size\ (n)}} \right|$$

The effect size can be classified as follows:

- effect size r less than 0.3 → small effect
- effect size r between 0.3 and 0.5 → medium effect
- effect size r greater than 0.5 → large effect

Mean IC50 values and Standard deviations of each tested drug were determined using Excel. The images were created with BioRender 2021 and the graphs with Prism - GraphPad.

3 Results

3.1 Activity of tetracyclines

Activities of the tested tetracyclines can be seen in Table 3, where the activities in the three-day and the six-day assay against *P. falciparum* measured by the HRP2 ELISA and *P. knowlesi* measured by Sybr Green-I assay are displayed.

All tetracyclines tested in this study exhibited an IC50 in micromolar range in the three-day assay, some of them were active in the six-day assay, showing a second cycle/ slow onset of activity. The drugs tested for the first time against *P. falciparum* demeclocycline, sarecycline, lymecycline, omadacycline as well as the already previously tested drugs doxycycline (Table 7), minocycline and eravacycline showed a delayed death effect in nanomolar range against 3D7 as the fold difference between three and six days was at least 34 (threshold 15); demeclocycline, minocycline and eravacycline also showed a delayed death effect against A1H1, with a fold change of at least 41. Methacycline showed a delayed death effect in 3D7 and A1H1 with IC50s after six days in micromolar range. Only 3D7 was susceptible to meclocycline after six days. Eravacycline, being the most potent tetracycline assessed in these experiments, revealed the lowest IC50 in the six-day assay with 5.1 nM for 3D7 and 12.6 nM for A1H1. Interestingly, only minocycline showed a delayed death effect against Dd2 as the IC50 after six days was 15-fold lower than after three days. Chlortetracycline was inactive against all parasite strains at all time points.

Table 3 Activity of tetracyclines against asexual stages of P. falciparum (drug-sensitive line 3D7 and multi-drug resistant line Dd2) and P. knowlesi (A1H1), evaluated by HRP2 ELISA and Sybr Green-I assay, respectively: This table summarizes the activity of tetracyclines against asexual stages of P. falciparum (drug-sensitive 3D7 and multi-drug resistant Dd2 strains) and P. knowlesi (A1H1) measured by HRP2 ELISA and Sybr Green-I, respectively. The assays were performed for 3 days to assess fast-acting drug activity (activity against the first cycle of P. falciparum) and for 6 days to evaluate delayed activity (activity against the second cycle of P. falciparum). For comparability, the assay for P. knowlesi (A1H1) was conducted for the same duration (3 and 6 days). A high fold-change difference between the 3-day and 6-day results indicates a pronounced delayed death effect. Each assay was performed at least twice in duplicate to ensure reproducibility.

Drug	3D7 IC50 (nM) ±SD			Dd2 IC50 (nM) ±SD			A1H1 IC50 (nM) ±SD		
	3 days	6 days	Fold differences between 3 and 6 days	3 days	6 days	Fold differences between 3 and 6 days	3 days	6 days	Fold differences between 3 and 6 days
Demeclocycline	47399 ± 16889	410.1 ± 165	116	26534 ± 4709	13698 ± 5465	1.9	> 50000	386 ± 262	> 130
Methacycline	> 50000	3719 ± 3134	> 13	> 18000	> 50000	-	> 50000	2986 ± 948	> 17
Minocycline	9393 ± 4354	83.6 ± 25	112	4540 ± 3140	305.7 ± 337	15	5917 ± 1651	146 ± 172	41
Meclocycline	26247 ± 2030	291.7 ± 41	90	9937 ± 3690	12258 ± 7081	0.8	7693 ± 4386	1492 ± 547	5
Chlortetracycline	> 18000	33664 ± 8994	> 0.5	48461 ± 14173	> 18000	-	22198 ± 8825	8315 ± 2630	2.7
Lymecycline	> 18000	1124 ± 247	> 16	> 18000	25940 ± 7180	-	1562 ± 5350	1152 ± 226	1.4
Sarecycline	13379 ± 1053	271.9 ± 136	49	10489 ± 4793	6821 ± 2059	1.5	1870 ± 1014	920 ± 512	2
Omadacycline	8443 ± 618	247.8 ± 109	34	7891 ± 1438	4928 ± 1005	1.6	1156 ± 399	284.9 ± 57	4
Eravacycline	2514 ± 550	5.1 ± 1.1	493	2926 ± 688	2124 ± 1252	1.4	2325 ± 1529	12.6 ± 11	185

3.2 Activity of macrolides

The macrolides showed a heterogenous activity profile against *Plasmodium* spp. Borrelidin and boromycin showed a fast onset of action (highly active after three days) with IC_{50} at single digit nanomolar range against *P. falciparum* strain 3D7 and Dd2 and *P. knowlesi* strain A1H1 (Figure 4 and 5 and Table 4). Josamycin displayed a slow effect against 3D7 and A1H1 with a fold difference between three and six days of 90 and 145 respectively but was inactive against Dd2 at all time points. Oleandomycin and troleandomycin were inactive, at both time points against all parasite strains (Table 4).

Table 4 Activity of macrolides Activity of macrolides against asexual stages of P. falciparum (drug-sensitive line 3D7 and multi-drug resistant line Dd2) and P. knowlesi (A1H1), evaluated by HRP2 ELISA and Sybr Green-I assay, respectively: This table summarizes the activity of macrolides against the asexual stages of P. falciparum (drug-sensitive 3D7 and multi-drug resistant Dd2 strains) and P. knowlesi (A1H1) measured by HRP2 ELISA and Sybr Green-I assays, respectively. The assays were conducted for 3 days to assess fast-acting drug activity (activity against the first cycle of P. falciparum) and for 6 days to evaluate delayed activity (activity against the second cycle of P. falciparum). For comparability, the assay for P. knowlesi (A1H1) was performed for the same durations (3 and 6 days). A significant fold-change difference between the 3-day and 6-day results indicates a pronounced delayed death effect. Each assay was performed at least twice in duplicate to ensure reproducibility.

Drug	3D7 IC50 (nM) ±SD		Dd2 IC50 (nM) ±SD		A1H1 IC50 (nM) ±SD	
	3 days	6 days	3 days	6 days	3 days	6 days
Borrelidin	0.6 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	2.7 ± 1	1.3 ± 0.5
Boromycin	0.9 ± 0.1	1.0 ± 0.8	0.7 ±0.4	0.5 ±0.3	6 ± 3.5	3.4 ± 1.4
Josamycin	34708 ± 7737	387.8 ± 185	15309 ± 1870	38235 ± 7081	23890 ± 9943	164.5 ± 147
Oleandomycin	8186 ± 3851	22075 ± 10527	> 18000	14245 ± 9750	27098 ± 15101	3916 ± 2766
Troleandomycin	> 37000	34839 ± 10109	10775 ± 2488	40624 ± 14778	45391 ± 27930	2817 ± 1974

For our most active substances Boromycin and Borrelidin, we also calculated the IC90 (Table 5), which are close to the IC50, an indication of a steep dose-reponse curve and pronounced antiplasmodial activity with drug concentrations in the nanomolar range.

Table 5 IC50 and IC90 values of boroycin and borrelidin asexual stages of P. falciparum (3D7): This table presents the mean 50% inhibitory concentrations (IC_{50}) and 90% inhibitory concentrations (IC_{90}), including standard deviations (±SD), of the highly active compounds boromycin and borrelidin against asexual stages of P. falciparum (drug-sensitive strain 3D7). Drug activity was assessed using the HRP2 ELISA method. Each assay was performed at least twice in duplicate to ensure reproducibility.

Drug	**3 days**		**6 days**	
3D7	IC50 (nM)	IC90 (nM)	IC50 (nM)	IC90 (nM)
Borrelidin	0.6 ± 0.1	0.9± 0.3	0.3 ± 0.2	0.3± 0.03
Boromycin	0.9 ± 0.1	1.8± 0.5	1.0 ± 0.8	2.2± 0.3

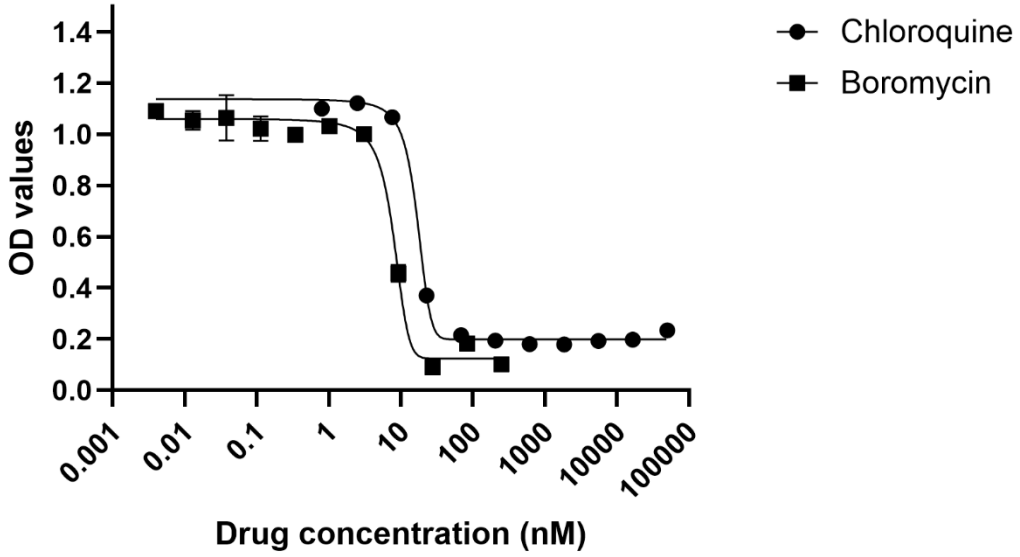

Figure 4 Comparison of activity of boromycin and chloroquineagainst asexual stages of P. falciparum in the standard growth inhibition assay: This figure presents a graphical comparison of the activity of boromycin and chloroquine against the asexual stages of Plasmodium falciparum 3D7. The data show optical density (OD) values relative to drug concentration after three days of incubation. Both boromycin and chloroquine exhibit comparable activity, with IC_{50} values in the nanomolar range. Each assay was performed at least twice in duplicate to ensure reproducibility.

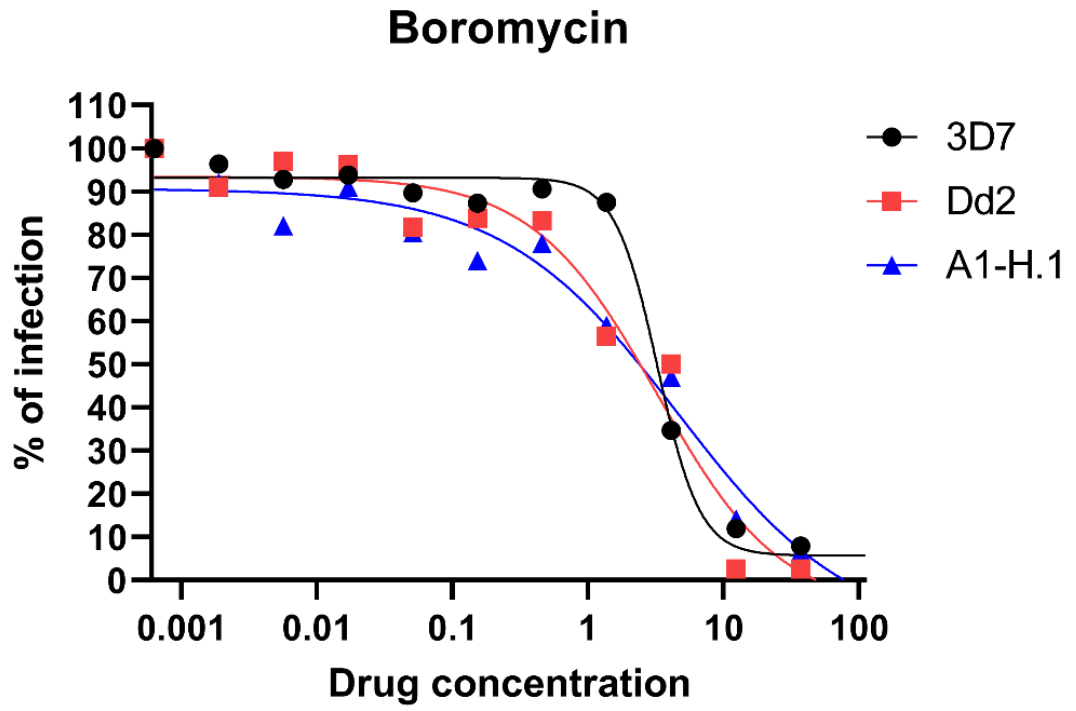

Figure 5 Activity of boromycin in two P. falciparum strains (3D7 and Dd2) and in P. knowlesi A1H1: This figure illustrates the percentage of parasite survival relative to boromycin concentration after three days of incubation in P. falciparum 3D7, Dd2, and P. knowlesi A1H1. The graph demonstrates that boromycin is active in the nanomolar (nM) range, exhibiting a rapid onset of action in both Plasmodium species. Each assay was performed at least twice in duplicate to ensure reliability.

3.3 Activity of nitroimidazoles and aminoglycosides

All parasite strains tested were unaffected by nitroimidazoles and aminoglycosides at both time points. Either the IC50 was higher than the highest concentration tested (111 µM) or inhibition could only be detected at the highest concentration, which makes calculation of an IC50 unfeasible (IC50 > 37 µM) (Table 6).

Table 6 Activity of nitroimidazoles and aminoglycosides against asexual stages of P. falciparum (drug-sensitive line 3D7 and multi-drug resistant line Dd2) evaluated by HRP2 ELISA and asexual stages of P. knowlesi (A1H1) measured by Sybr Green-I assay: This table summarizes the activity of nitroimidazoles (nimorazole, metronidazole, tinidazole) and aminoglycosides (kanamycin, gentamicin, streptomycin) against the asexual stages of P. falciparum (drug-sensitive 3D7 and multi-drug resistant Dd2 strains) and P. knowlesi (A1H1) measured by HRP2 ELISA and Sybr Green-I assays, respectively. The assays were performed for 3 days to assess fast-acting drug activity (activity against the first cycle of P. falciparum) and for 6 days to evaluate delayed activity (activity against the second cycle of P. falciparum). For comparability, the assay for P. knowlesi (A1H1) was conducted for the same durations (3 and 6 days). Each assay was performed at least twice in duplicate to ensure reproducibility. The results indicate that nitroimidazoles and aminoglycosides do not exhibit significant activity in the standard growth inhibition assay against P. falciparum 3D7, Dd2, or P. knowlesi A1H1.

Drug	**3D7 IC50 (nM) ±SD**		**Dd2 IC50 (nM) ±SD**		**A1H1 IC50 (nM) ±SD**	
	3 days	6 days	3 days	6 days	3 days	6 days
Tinidazole	> 111000	> 111000	> 111000	> 111000	> 111000	> 111000
Nimorazole	> 111000	> 37000	> 111000	> 111000	> 111000	> 111000
Metronidazole	> 111000	> 37000	> 111000	> 111000	> 111000	> 111000
Kanamycin	> 111000	> 111000	> 111000	> 111000	> 111000	> 111000
Streptomycin	> 111000	> 111000	> 111000	> 111000	> 111000	> 111000
Gentamicin	> 111000	> 111000	> 111000	> 111000	> 111000	> 111000

3.4 Controls

As expected 3D7 and A1H1 were sensitive to chloroquine and quinine while Dd2 was not susceptible to both drugs. Chlorotonil A demonstrated IC50s in nanomolar range for both time points and both *Plasmodium* spp., ivermectin for both time points in *P. falciparum*. The three-day assay control antibiotic doxycycline showed a delayed death effect in 3D7 (fold difference between three and six days of 34) but not in Dd2. The six-day assay reference antibiotic clindamycin demonstrated a delayed death effect with nanomolar IC50s after several cycles in both *Plasmodium* spp. (Table 7).

Table 7 Activity of control drugs against asexual stages of P. falciparum (drug-sensitive line 3D7 and multi-drug resistant line Dd2) and P. knowlesi (A1H1), evaluated by HRP2 ELISA and Sybr Green-I assay, respectively: The table reports mean 50% inhibitory concentrations (IC_{50}) in nM, including standard deviations (±SD). The assays were conducted for 3 days to assess fast-acting drug activity (activity against the first cycle of P. falciparum) and for 6 days to evaluate delayed activity (activity against the second cycle of P. falciparum). For comparability, the assay for P. knowlesi (A1H1) was performed for the same durations (3 and 6 days). A significant fold-change difference between the 3-day and 6-day results indicates a pronounced delayed death effect. Each assay was performed at least twice in duplicate to ensure reproducibility.

Drug	**3D7 IC50 (nM) ±SD**		**Dd2 IC50 (nM) ±SD**		**A1H1 IC50 (nM) ±SD**	
	3 days	6 days	3 days	6 days	3 days	6 days
Chloroquine	8.4 ± 4.9	10.5 ± 4.8	302.6 ± 119	561.1 ± 212	14.6 ± 9.3	
Quinine	-	16.8 ± 3.7	295 ± 49	417 ± 168	36.5 ± 6.5	33 ± 1.2
Chlorotonil A	18 ± 5.7	20.6 ± 6.7	16.9 ± 13	33 ± 19	33.6 ± 12.5	40 ± 13
Ivermectin	-	637 ± 305	553 ± 201	643 ± 85	1541 ± 324	2402 ± 308
Doxycycline	9579 ± 2756	385.7 ± 292	5067 ± 1802	6930 ± 1323	1849 ± 1035	663 ± 508
Clindamycin	> 37000	6 ± 3.4	> 111000	4.7 ± 2.5	77.2 ± 64	15 ± 12

3.5 Characterization of the antiplasmodial activity

3.5.1 Activity on the apicoplast

In the previous chapters, a delayed death effect was observed for some tetracyclines and macrolides, and a fast onset of activity was observed for borrelidin and boromycin. As stated in the methods (*2.8.1 IPP supplementation assay*), an incubation with IPP was performed to assess whether the apicoplast is the main target of these antibiotics. Parasites 3D7 and A1H1 treated with minocycline, josamycin, eravacycline and clindamycin were rescued by supplementation with IPP as the IC50s were at least 40 times higher than the control without IPP supplementation (see Table 8, Figure 7). *P. falciparum* 3D7 treated with the most active macrolides borrelidin and boromycin could not be rescued by IPP supplementation. IC50 with IPP addition were even lower than the controls.

Table 8 Results of the IPP supplementation assay in P. falciparum (3D7) and P. knowlesi (A1H1): This table presents mean 50% inhibitory concentrations (IC_{50}) and standard deviations (±SD) in nM after six days of incubation with IPP supplementation compared to parasites incubated with the drug and without IPP (controls), tested in P. falciparum (3D7) using HRP2 ELISA and in P. knowlesi (A1H1) using Sybr Green-I. The IPP supplementation assay was performed at least twice in duplicate to ensure reproducibility. The results show that parasites treated with minocycline, josamycin, eravacycline, and clindamycin were rescued by IPP supplementation, as indicated by IC_{50} values that were at least 40-fold higher compared to the respective controls without IPP (see Table 8, Figure 7). In contrast, IPP supplementation did not rescue P. falciparum 3D7 treated with the fast-acting macrolides borrelidin and boromycin, indicating that their mode of action is independent of the apicoplast.

Drug	**3D7**		**A1H1**	
	IPP	**Control**	**IPP**	**control**
Minocycline	6521 ± 1259	84 ± 25	5885 ± 3410	146 ± 172
Josamycin	29469 ± 9478	388 ± 185	108865 ± 103778	165 ± 147
Clindamycin	> 22 µM	6 ± 3.4	> 37 µM	15 ± 12
Borrelidin	0.132 ± 0.06	0.3 ± 0.22	-	-
Boromycin	0.313 ± 0.13	1 ± 0.75	-	-
Eravacycline	-	-	2410 ± 836	13 ± 11

To objectively evaluate the significance of differences in IC_{50} values with and without IPP supplementation, the non-parametric Mann–Whitney U test was performed (Table 10). This test was chosen because it does not require the assumption of normal distribution and is therefore appropriate for small sample sizes, where normality cannot be reliably assessed.

Here, a p-value < 0.05 supports the apicoplast as the primary drug target of clindamycin, eravacycline, minocycline, and josamycin, all of which exhibit a delayed death effect. Furthermore, the data suggest that IPP supplementation reverses the delayed death effect of these antibiotics in both parasite species, reinforcing the apicoplast as the target also in *P. knowlesi* A1H1.

The greatest difference in IC_{50} values was observed for the reference drug clindamycin, with a more than 2,000-fold change upon IPP supplementation. In contrast, boromycin and borrelidin had similar activities with and without IPP (both p > 0.05), suggesting that these fast-acting compounds do not primarily target the apicoplast during the asexual erythrocytic stage of the parasite (Table 8, Figure 7).

In addition to *p*-values, effect sizes were calculated to estimate the magnitude of the differences observed. Effect size (*r*) was computed using the formula $r = Z / \sqrt{N}$, where Z is the standardized test statistic and N is the total number of observations. An *r* value > 0.5 is generally interpreted as a large effect (see Table 9).

Table 9 Statistical evaluation of IPP supplementation effects using the Mann-Whitney U test: This table summarizes the statistical analysis of IPP supplementation effects in 6-day drug assays for P. falciparum 3D7 and P. knowlesi A1H1. The Mann-Whitney U test was performed using SPSS to determine whether IPP addition significantly increased the IC_{50} values, indicating a delayed death phenotype and apicoplast-dependent drug activity. A significant effect was defined by a p-value < 0.05 and an effect size (r) > 0.5. Clindamycin, eravacycline, minocycline, and josamycin showed statistically significant increases in IC_{50} upon IPP supplementation, confirming delayed death and the apicoplast as their primary target. In contrast, boromycin and borrelidin did not show significant changes, suggesting an apicoplast-independent mode of action.

Mann-Whitney-U test

Drugs tested with and without IPP supplementation		p-value	Effect size
3D7	Minocycline	0.004	0.83
	Josamycin	0.004	0.78
	Clindamycin	<0.01	0.85
	Boromycin	0.24	0.34
	Borrelidin	0.262	0.29
A1H1	Minocycline	0.009	0.63
	Josamycin	0.009	0.72
	Clindamycin	<0.01	0.63
	Eravacycline	0.004	0.78

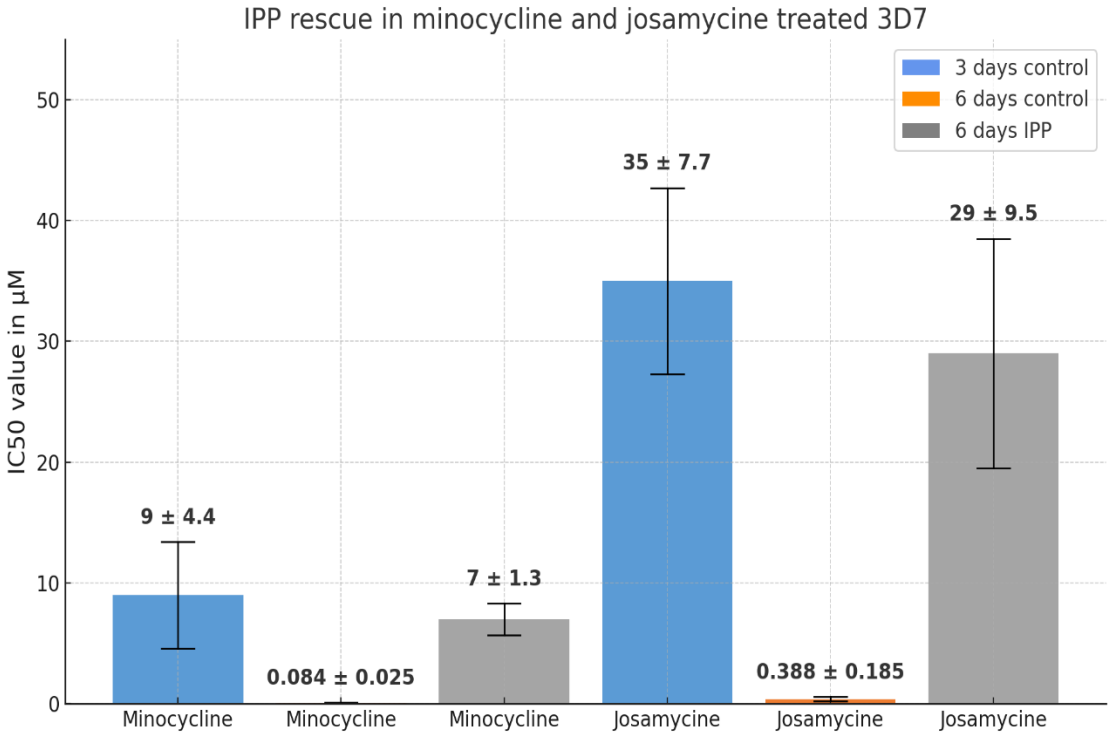

Figure 6 IPP rescue in minocycline and josamycin treated 3D7: This figure presents a growth inhibition assay of minocycline and josamycin against the asexual stages of P. falciparum 3D7, performed with and without IPP supplementation. Parasites supplemented with IPP (grey bars) show similar IC_{50} values to the control group without IPP after three days (blue bars), indicating fast off-target activity. After six days, the IC_{50} values without IPP (orange bars) are significantly lower, confirming the apicoplast as the target for these drugs, with a delayed death effect. The numbers above the bars represent the mean IC_{50} values in µM. Each assay was performed at least twice in duplicate. Standard deviations are provided in Tables 3, 4, and 8.

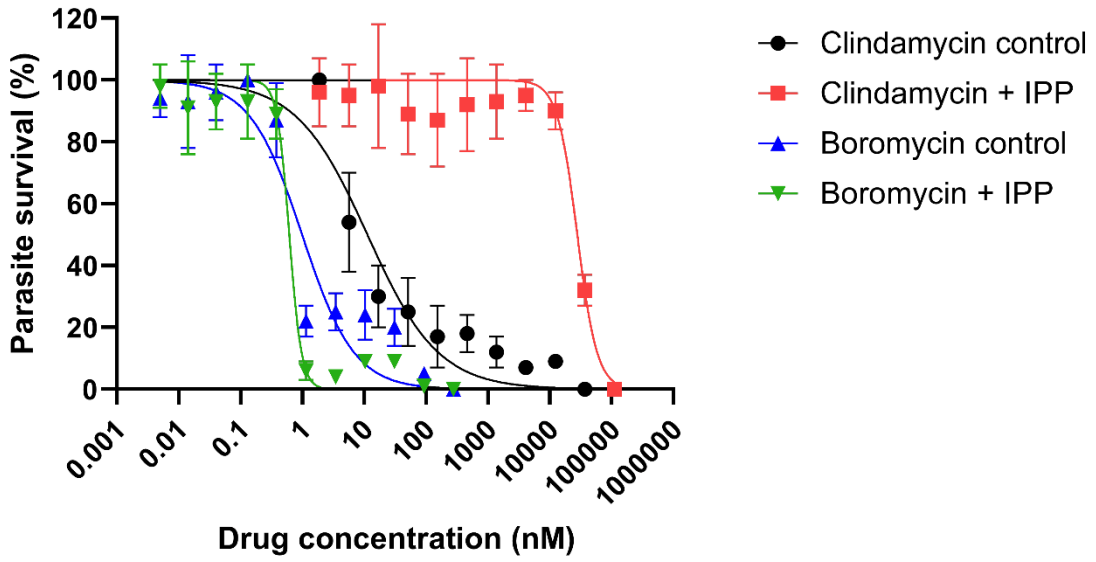

Figure 7 IPP assay: Clindamycin and Boromycin after six days of incubation in P. falciparum with and without IPP supplementation: This figure shows the percentage of parasite survival relative to drug concentration after six days of incubation with P. falciparum, comparing treatments with and without IPP supplementation. The survival curves of boromycin (with and without IPP addition) closely resembles that of minocycline without IPP, indicating consistent drug activity. In contrast, minocycline with IPP supplementation only reduces parasite survival at very high drug concentrations, suggesting that the activity of the drug is suppressed by IPP. This supports the conclusion that the apicoplast is the primary drug target. The IPP supplementation assay was performed at least twice in duplicate to ensure reproducibility.

3.5.2 Ionophoric activity

To assess boromycin´s ionophoric activity, 3D7 drug sensitivity assays of boromycin and chloroquine (reference drug) were repeated with supplementation of 220 µg/ml KCl or $MgCl_2$ (Table 11). The IC50 of boromycin increased three times with the addition of KCL, while no IC50 increase was observed for chloroquine. In contrast, supplementation of $MgCl_2$ resulted in a three- to five-fold increase of both drug activities as IC50s were lower than in the control assay. Although changes in the IC50 can be recognised, all IC50s are too low to assume that the addition of KCl or $MgCl_2$ cancel the effect of Boromycin.

Table 10 Ionophoric activity by KCl and $MgCl_2$ supplementation in P. falciparum 3D7 drug assays: This table presents the results of drug sensitivity assays in P. falciparum 3D7 with the addition of KCl (220 µg/mL) or $MgCl_2$ (220 µg/mL) to test for potential ionophoric activity of boromycin and the control compound chloroquine compared to parasites incubated with the drug and without KCL or $MgCl_2$ addition. The assays were performed at least twice in duplicate. IC_{50} values remained in the nanomolar range following salt supplementation for both compounds, indicating that ion transport disruption is unlikely to be the primary mode of action of boromycin.

Drug	**control IC50 (nM) ±SD**	**KCl supplementation IC50 (nM) ±SD**	**MgCl2 supplementation IC50 (nM) ±SD**
Boromycin	0.7 ± 0.1	2.1 ± 0.2	0.2 ± 0
Chloroquine	12 ± 0.8	11.6 ± 1.4	2.2 ± 0

3.5.3 Stage specific assay

To profile stage specificity and morphological changes of boromycin 1nM treated parasites, we performed a stage specific assay. There are several important reasons to assess stage specificity in antimalarial drug evaluation. First, activity against specific life cycle stages of the parasite can provide insight into the compound's mode of action, as distinct metabolic pathways are active at different developmental stages. Early intervention at the ring stage—the initial intraerythrocytic form of *Plasmodium*—is particularly valuable, as it occurs prior to parasite replication and thus offers an opportunity to limit disease progression. Furthermore, rapid elimination of ring-stage parasites can prevent their maturation into mature stages (trophozoites and schizonts), which are associated with increased virulence and inflammatory responses. A compound capable of targeting ring stages exhibits a unique and complementary mechanism of action, making it especially beneficial in the context of combination therapies. Among currently used antimalarial drugs, only artemisinins demonstrate activity across all asexual blood stages, including the ring stage. In contrast, other clinically used compounds—particularly aminoquinolines—primarily act against more mature stages, such as trophozoites. Untreated parasites showed the expected life cycle of 48 hours. When parasites were treated as rings (Figure 8 B1), trophozoites (Figure 8 B2), or schizonts (Figure 8 B3) with boromycin, the further development of the parasites was arrested and appeared pyknotic after six hours. Thus, boromycin was found to be effective at multiple life cycle stages and to produce the same appearance.

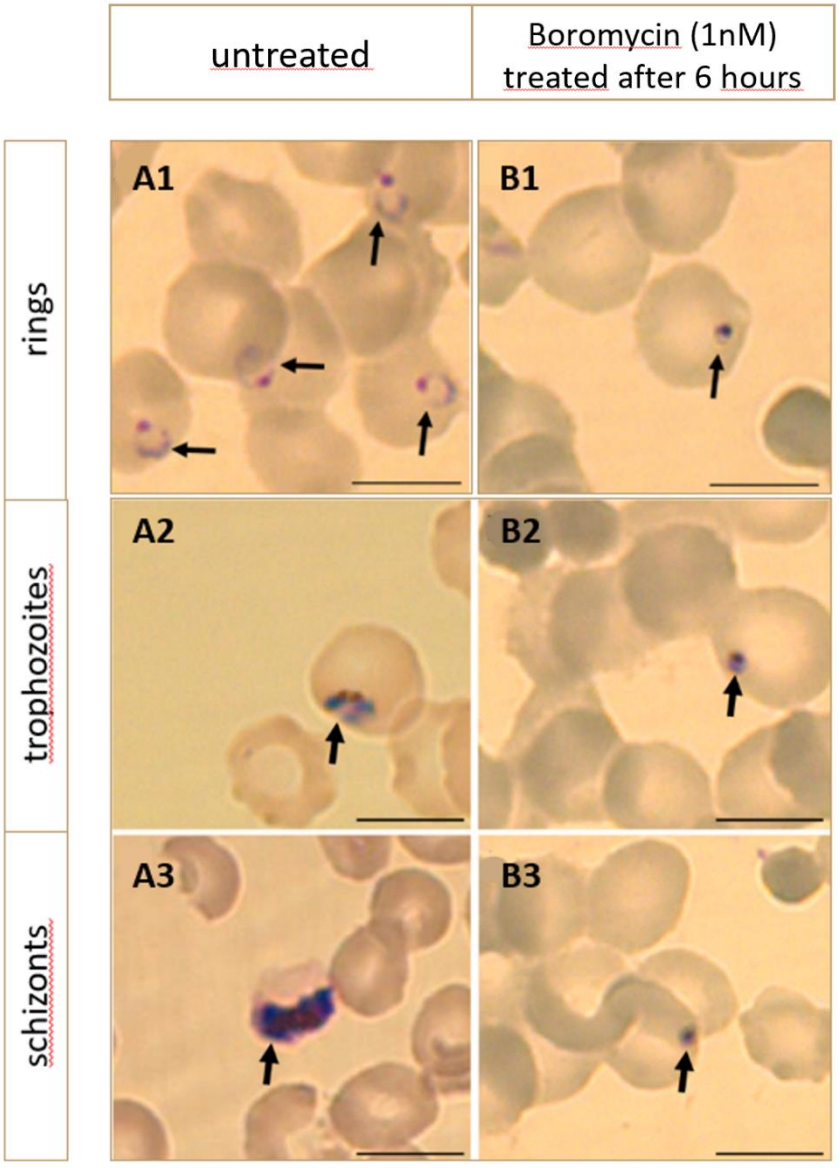

Figure 8 Stage specific assay: P. falciparum 3D7 was incubated with and without boromycin, and thin blood smears were prepared at several time points to further investigate the mode of action, focusing on potential stage specificity. This figure shows representative micrographs of P. falciparum 3D7-infected erythrocytes stained with Giemsa: (A) The first column shows parasites at time point 0 h: (A1) early rings, (A2) trophozoites, (A3) schizonts. (B) The second column shows parasites after 6 h of incubation with 1 nM boromycin: (B1) treated rings, (B2) treated trophozoites, (B3) treated schizonts. The representative micrographs of boromycin-treated parasites exhibit a pyknotic appearance in all parasite stages, demonstrating a rapid onset of action after 6 hours. Arrows indicate parasites. Scale bar: 10 µm. Figure adapted from our publication (191).

In addition to assessing the rapid onset of action, we aimed to monitor the progression of the *Plasmodium falciparum* intraerythrocytic life cycle over a 48-hour period. As shown in the Table 11 and Figure 9, a proportion of parasites continue to develop through the cycle, while others—particularly following the ring stage, but also after other stages—exhibit a pyknotic morphology, indicative of parasite death. After 12 hours of incubation of ring-stage parasites with boromycin, approximately 60% exhibit signs of death. In the stage-specific assay, 48 hours of incubation resulted in about 50% parasite death, which is consistent with the IC_{50} values obtained in the drug sensitivity assays. Although boromycin shows some activity against other developmental stages, it demonstrates a marked specificity for the ring stage. This selectivity is particularly advantageous given the aforementioned importance of targeting early parasite stages.

Table 11 Percentage of stage distribution of P. falciparum 3D7 after boromycin treatment at different time points: This table shows the percentage distribution of P. falciparum asexual stages (rings, trophozoites, schizonts) following treatment with boromycin over time, based on microscopic evaluation of Giemsa-stained blood smears. Three parallel experiments were conducted, each starting with synchronized parasites at different developmental stages: Table 1 begins with rings, Table 2 starts with trophozoites, and Table 3 initiates with schizonts. The assay was repeated twice for reproducibility. The results indicate that boromycin exhibits multi-stage activity, as damaged or dying parasites were observed at all developmental stages and time points; however, its activity is most pronounced against ring-stage parasites. Nevertheless, a subset of parasites was able to progress normally through the intraerythrocytic developmental cycle.

Start with rings								
Time point	Rings (%)		Trophozoites (%)		Schizonts (%)		Dead (%)	
	Boromycin	Control	Boromycin	Control	Boromycin	Control	Boromycin	control
0	**93**	93	7	7	0	0	0	0
12	**36**	65	6	35	0	0	**58**	0
24	**36**	0	**28**	100	0	0	**36**	0
36	11	12	0	61	**30**	27	**59**	0
48	**43**	90	1	10	0	0	**56**	0

Start with trophozoites								
Time point	Rings (%)		Trophozoites (%)		Schizonts (%)		Dead (%)	
	Boromycin	Control	Boromycin	Control	Boromycin	Control	Boromycin	control
0	11	11	**82**	82	7	7	0	0
12	8	5	29	21	**55**	74	8	0
24	**61**	86	0	3	8	11	**31**	0
36	**74**	95	5	5	0	0	**21**	0
48	**32**	12	**33**	82	6	6	**30**	0

Start with schizonts								
Time point	Rings (%)		Trophozoites (%)		Schizonts (%)		Dead (%)	
	Boromycin	Control	Boromycin	Control	Boromycin	Control	Boromycin	control
0	0	0	0	0	**100**	100	0	0
12	**64**	77	0	0	19	23	**17**	0
24	**89**	100	0	0	0	0	**11**	0
36	**37**	10	**44**	84	0	6	**19**	0
48	0	2	**70**	10	7	88	**23**	0

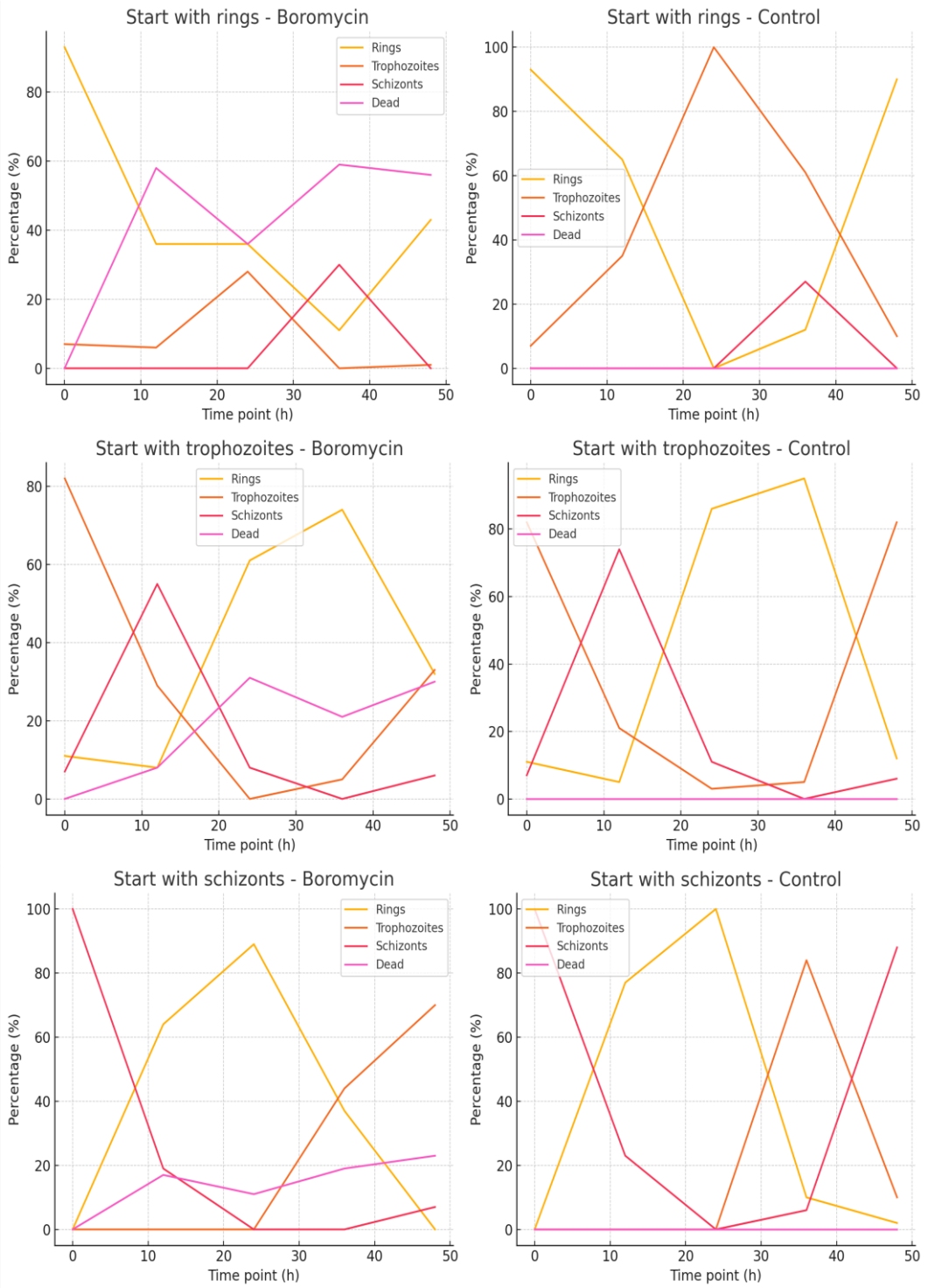

Figure 9 *Percentage of stage distribution of P. falciparum 3D7 after boromycin treatment at different time points shown graphically: Three parallel experiments were conducted, each starting with synchronized parasites at different developmental stages: The first graph begins with rings, the second with trophozoites, and the third initiates with schizonts. The assay was repeated twice for reproducibility. The results indicate that boromycin exhibits multi-stage activity, as damaged or dying parasites were observed at all developmental stages and time points; however, its activity is most pronounced against ring-stage parasites. Nevertheless, a subset of parasites was able to progress normally through the intraerythrocytic developmental cycle.*

3.6 Cytotoxicity of boromycin on HepG2 cells

To further investigate the cytotoxicity profile of boromycin, its effect on HepG2 cells was examined. As illustrated in Table 13, boromycin showed low cytotoxicity (1250.5 µM ± 230) in the HepG2 cytotoxicity assay, consequently, the selectivity index was > 200,000 for *P. knowlesi* and even > 1,250,000 for *P. falciparum*, once the IC_{50} for the parasites were ~ 6 nM and ~ 2 nM respectively. The reference drug chloroquine displayed a lower IC_{50} against HepG2 cells, therefore less specificity.

Table 12 Results of the cytotoxicity assay for boromycin: This table presents the mean 50% inhibitory concentrations (IC50) and standard deviations (±SD) in µM of boromycin and the control drug chloroquine in the HepG2 cytotoxicity assay, as well as the selectivity index for P. falciparum and P. knowlesi. The high selectivity index for boromycin in both parasite species indicates its low cytotoxicity to human HepG2 cells, demonstrating a favorable therapeutic index. The assay was performed twice in duplicate.

Drug	***HepG2 IC50 (µM) ±SD***	***Selectivity index (P. falciparum)***	***Selectivity index (P. knowlesi)***
Boromycin	1250.5 ± 230	> 1,250,000	> 200,000
Chloroquine	153.4 µM ± 52.9	18,200	10,600

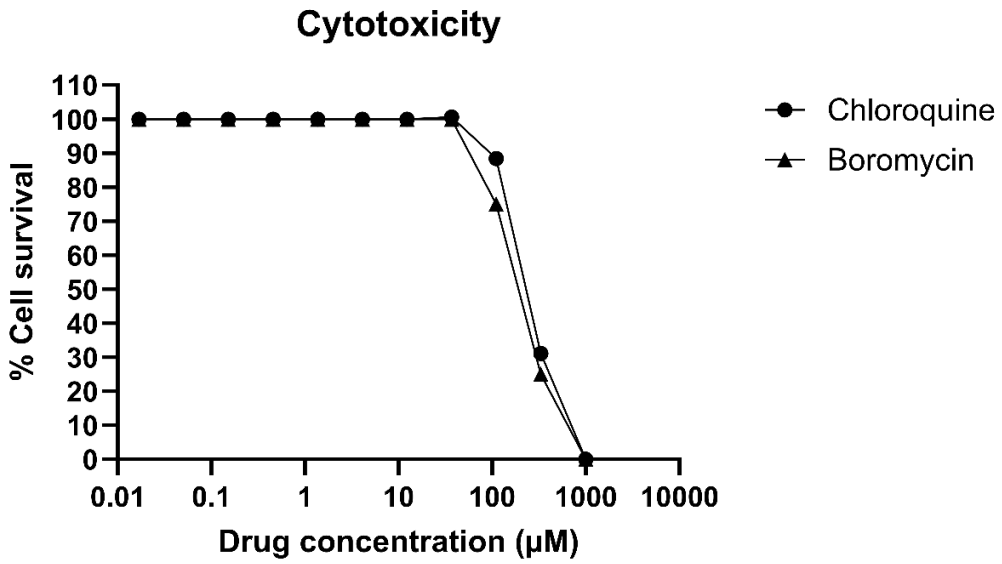

Figure 10 Cytotoxicity of Boromycin and Chloroquine in HEPG2 cells: This figure presents the percentage of survival of HEPG2 cells relative to the concentration of boromycin and chloroquine. The data show that boromycin induces cytotoxicity in HEPG2 cells comparable to chloroquine. Due to its relatively low cytotoxicity against HEPG2 cells, boromycin emerges as a promising drug candidate for further investigation. The assay was performed twice in duplicate to ensure reproducibility.

3.7 Comparison of different species and strains

3.7.1 Difference between 3D7 and Dd2

Most antibiotics exhibited similar activity (within a three-fold difference) in chloroquine-sensitive 3D7 and chloroquine resistant Dd2 as can be seen from the previous tables (Tables 3-7). High and fast activity with IC50s in single digit nanomolar range of the two most active drugs boromycin and borrelidin suggest no cross-resistance for the parasite line Dd2. In contrast to the results of the 3D7 drug assays, josamycin, demeclocycline, methacycline, lymecycline, sarecycline, omadacycline and eravacycline were less active against Dd2 after six days and consequently the fold difference between three and six days was not high enough (< 15) to define a late onset of action.

3.7.2 Difference between Plasmodium knowlesi (A1H1) and Plasmodium falciparum (3D7)

In general, antibiotics inactive against *P. falciparum* (oleandomycin, troleandomycin, chlortetracycline, nimorazole, tinidazole, metronidazole, kanamycin, streptomycin, gentamicin) were also inactive against *P. knowlesi* (Tables 3-7). The most active macrolides boromycin and borrelidin exhibited slightly higher IC50s in *P. knowlesi* but were still active in nanomolar range (Table 4). Likewise, the fast-acting control drugs (chloroquine, chlorotonil A, ivermectin, quinine) also had similar (within a three-fold difference) IC50s for both parasite species (Table 7).

Most of the drugs with a delayed death effect in 3D7 also exhibited more activity at the second time point in *P. knowlesi* A1H1 (meclocycline, omadacycline, demeclocycline, minocycline, eravacycline, josamycin, doxycycline, clindamycin). Except of meclocycline, omadacycline, doxycycline and clindamycin, the fold difference between three and six days for *P. knowlesi* A1H1 was higher than 20, which indicates a delayed death phenotype following treatment.

Regarding the methods used, it should be mentioned that *P. knowlesi* showed similar growth rate in culture supplemented with human serum or Albumax II (see one example dilution series in Table 14).

Table 13 Exemplary parasite culture dilution of P. knowlesi cultured in different media : This table shows the dilution of P. knowlesi cultured in various media compositions: 5% human AB serum, 20% v/v Albumax II solution, and 10% v/v Albumax II solution. P. knowlesi demonstrated good growth in all tested media, indicating that the parasite can grow under varying nutrient conditions. These media conditions were used to assess the growth and viability of P. knowlesi.

Time points of dilution		0 h	48 h	96 h	168 h	216 h
Ratio of dilution	5% serum	1:5	1:10	1:10	1:10	1:5
	20 % Albumax	1:5	1:5	1:20	1:10	1:2
	10 % Albumax	1:5	1:5	1:20	1:10	1:2

Nevertheless, only *P. knowlesi* parasite cultures in human serum could be synchronised using Nycodenz, as the cultures in Albumax II and Albumax I needed several days after synchronisation to return to their normal growth rate (defined as at least doubling of OD values between maximum drug concentration and negative control). Thus, for our internal SOP and the results presented in this thesis, it was decided that the parasites were cultured and synchronised in human serum but incubated in Albumax II for the experiments to produce similar conditions as for *P. falciparum* (166).

4 Discussion

The emergence of resistance to antimalarial therapies continues to challenge malaria control and eradication efforts. Expanding the portfolio of effective drugs is a key strategy to mitigate this threat (192, 193). One promising approach is the repurposing of existing antibiotics, which offers a cost-effective and time-efficient alternative to de novo drug development—especially important given the limited investment in neglected tropical diseases. In this study, we assessed a panel of clinically approved or investigational antibiotics for their in vitro activity against *Plasmodium falciparum* and *Plasmodium knowlesi*. Several antibiotics, such as doxycycline and clindamycin, are already in use for malaria prophylaxis or treatment, demonstrating the viability of this strategy (38).

4.1 Tetracyclines

Tetracyclines are a well-established class of slow-acting antimalarial drugs (194). Our *in vitro* findings confirmed previously reported activity for eravacycline (49), minocycline and doxycycline (194). In contrast, no measurable in vitro activity was observed for chlortetracycline after either three or six days of incubation, despite earlier reports of clinical activity against *P. falciparum* and *P. vivax* in uncomplicated malaria (54, 55, 173). Notably, those studies reported limited efficacy, with no cures achieved and only slow parasite clearance. The discrepancy between our findings and earlier data may be attributed to fundamental differences in study design—namely, in vitro versus in vivo systems. Additionally, chlortetracycline demonstrated species-specific variability in historical studies, showing greater efficacy against *P. cathemerium* and *P. berghei* compared to *P. gallinaceum*, suggesting that it may lack activity against the specific *P. falciparum* strains (Dd2, 3D7) or *P. knowlesi* used in our study. Given the small sample sizes in the historical clinical studies, these findings should be interpreted with caution.

Several tetracyclines newly tested in this study - including demeclocycline, sarecycline, and omadacycline - exhibited characteristics of the "delayed death effect", a phenomenon associated with antibiotics that target the apicoplast (48, 49). Although no standardized threshold currently exists to define this effect, our

data revealed a clear dichotomy: compounds either showed a minimal difference in IC_{50} between day 3 and day 6 (<5-fold) or a pronounced increase (>30-fold). Methacycline and lymecycline demonstrated differences >17-fold and >16-fold, respectively, although no IC_{50} could be calculated at day 3 due to lack of measurable activity (IC_{50} >50,000 nM and >18,000 nM). Based on this observation, we propose a provisional threshold of >15-fold difference to classify delayed death activity. Previous work by Uddin et al. referenced this phenomenon without a quantitative definition but mentioned a >100-fold reduction in IC_{50} for azithromycin between the first and second cycle (31). The establishment of a standardized definition would greatly enhance comparability between studies and clarify the classification of slow-acting compounds.

Among the newly tested tetracyclines, none demonstrated activity comparable to next-generation compounds such as tigecycline (48) or eravacycline (49). This observation supports the hypothesis that specific chemical modifications—particularly at the C9 position of the D-ring—may enhance antiplasmodial activity while simultaneously improving resistance profiles in bacterial pathogens. Interestingly, omadacycline, which shares similar structural features, did not exhibit the same potency, a divergence also noted in studies on *Rickettsia* spp. (195). These findings suggest that subtle differences in molecular structure can significantly affect activity and warrant further investigation into structure–activity relationships in *Plasmodium* spp.

4.2 Macrolides

Macrolides, the second antibiotic class evaluated, can be broadly categorized into slow-acting and fast-acting compounds. The phenomenon of the delayed death effect - described in detail in section 4.1 for tetracyclines - is also well documented for several macrolides, including clarithromycin, azithromycin, and erythromycin (31), and was similarly observed for josamycin, which we tested for the first time. Uddin et al. further characterized this phenomenon and confirmed apicoplast targeting for various compounds via isopentenyl pyrophosphate (IPP) rescue assays. Using a comparable approach, we verified apicoplast

dependency for minocycline and eravacycline (previously shown for *P. falciparum* (49)) as well as for josamycin in both *P. falciparum* and *P. knowlesi*.

The delayed onset of activity observed in apicoplast-targeting drugs can be beneficial in combination therapy, where slow-acting compounds complement fast-acting partners by clearing residual parasites. This extended activity helps prevent recrudescence and may delay or reduce the emergence of resistance (111).

4.2.1 Fast-acting Macrolides: Borrelidin and Boromycin

In contrast to slow-acting macrolides, borrelidin and boromycin exhibited potent activity in the nanomolar range during the first replication cycle (three-day assay), an essential property for rapidly reducing parasitemia and mitigating complications. For borrelidin, in vitro IC_{50} values of ~1.9 nM against *P. falciparum* (K1 strain), and in vivo efficacy in *P. yoelii* and *P. berghei* (ED_{50} = 0.07 mg/kg and 0.18 mg/kg, respectively) have been documented (77, 195). Similarly, boromycin demonstrated in vivo activity against *P. berghei* (ED_{50} = 2.2 mg/kg) and *P. gallinaceum* in birds (ED_{50} = 7 mg/kg) (107).

While borrelidin shows low cytotoxicity in MRC-5 cells (77), in vivo toxicity has been reported, including weight loss and high mortality in treated mice. Several sources classify it as too toxic for therapeutic use (79-82, 196-198), likely due to its inhibition of both prokaryotic and eukaryotic threonyl-tRNA synthetases (199, 200). As a result, further evaluation of borrelidin was not pursued in our study. However, future investigations might consider less toxic analogues (82, 198, 201).

4.2.2 Boromycin: Human cytotoxicity and properties in Plasmodium spp.

Boromycin demonstrated a high selectivity index (>200,000) against HepG2 cells, aligning with prior reports of low cytotoxicity based on assays such as MTS and ATP quantification across various cell lines (HFF, HCT-8 and vero cells) (104, 106, 183, 202). Despite this promising in vitro safety profile, toxicity assessments limited to isolated cell lines represent a constraint. Potential effects on hematopoietic cells, particularly important during blood-stage malaria as host cell

and as mediator of immune responses, remain unexplored and warrant further investigation. Notably, in vivo data report an LD_{50} of 180 mg/kg in mice (203), supporting further preclinical evaluation.

Importantly, boromycin displayed consistent IC_{50} values across the drug-resistant *P. falciparum* strain Dd2, the reference strain 3D7, and *P. knowlesi* A1H1. This indicates a lack of cross-resistance with established antimalarials such as chloroquine, pyrimethamine, and mefloquine. These results are in line with previously published data from our group demonstrating similar IC_{50}s for 7G8 and K1 strains (191). In the same study, boromycin also exhibited gametocytocidal activity surpassing the control methylene blue, highlighting its potential to block malaria transmission.

Another notable feature of boromycin as a potential antimalarial agent is its rapid onset of activity, a critical requirement for the treatment of severe malaria to prevent organ complications. Early intervention at the ring stage - the earliest intraerythrocytic form of the parasite - can be beneficial, as it prevents replication and enables the rapid elimination of parasites before they mature into later stages (trophozoites and schizonts).

In our stage-specific assays, parasites at the ring, trophozoite, and schizont stages exhibited a pyknotic morphology after just six hours of treatment with 1 nM boromycin (approximately the IC_{50}). Notably, boromycin displayed its highest activity against ring-stage parasites. Although a specific effect can be observed, a portion of the parasites completes their normal life cycle. This is due to the fact that we used boromycin at its IC_{50} concentration, at which, by definition, 50% of the parasites survive. While repeating this experiment with a higher drug concentration would be advisable, the initial concentration was deliberately selected to elucidate the drug's specific effect.

Among currently used antimalarial drugs, only artemisinins demonstrate activity across all asexual blood stages, including the ring stage. This rapid onset of action is also a defining advantage of artemisinin derivatives, the cornerstone of current artemisinin-based combination therapies (ACTs) (34, 204, 205). For example, in *Plasmodium falciparum* 3D7, the 72-hour IC_{50} for dihydroartemisinin

(1.5 nM) is achieved within 8 hours, underscoring its fast-acting profile (205). Artemisinin-sensitive *Plasmodium* spp. are characterized by a parasite clearance half-life of ≤ 5 hours, reflecting the swift efficacy of these compounds (158). A shared characteristic of boromycin and artemisinin derivatives is the induction of pyknotic parasite morphology, resembling Howell–Jolly body-like inclusions (158). In contrast, aminoquinoline antimalarials—such as chloroquine, quinine, amodiaquine, and lumefantrine—display stage-specific activity predominantly from the trophozoite stage onwards and are thus unable to induce a similarly rapid parasiticidal effect (206, 207).

These observations underscore the need for further investigations into the time-dependent IC_{50} dynamics and stage-specific activity. Complementary methods such as metabolomic profiling, as demonstrated by Murithi et al. (205), could provide more detailed mechanistic insights. A limitation of microscopy is its inability to clearly distinguish small trophozoites from nonviable parasites.

In light of its favorable pharmacological properties and transmission-blocking potential, boromycin represents a promising candidate for further preclinical development.

4.2.3 Boromycin: mode of action

To explore the mechanism of action, we employed two experimental approaches: IPP rescue to investigate apicoplast dependency and KCl supplementation to evaluate ionophoric activity. As expected, IPP failed to rescue boromycin-treated parasites, suggesting that the apicoplast is not the primary target in asexual blood stages. This finding contrasts with other antibiotics, such as fosmidomycin, which can be rescued by IPP despite rapid activity (25).

Boromycin is known to act as a potassium ionophore in Gram-positive bacteria, disrupting membrane potential by inducing potassium efflux (102). Gram-negative bacteria, which possess an outer membrane, are typically resistant because this additional lipid barrier prevents boromycin from entering the cytosol. Nevertheless, activity has been demonstrated against the gram-negative *Mycobacterium bovis*, which still allows sufficient uptake of the compound (104). In these studies, growth inhibition was reversed by potassium chloride (KCl)

supplementation: the addition of 20 mg/mL KCl reduced boromycin-induced growth inhibition to approximately 10 %, which counteracts boromycin's mechanism of action. The high extracellular potassium concentration inhibits further efflux of intracellular potassium ions, thereby maintaining membrane potential and allowing potassium-dependent processes, such as protein and RNA synthesis, to continue.

To test for similar effects in *Plasmodium* spp., we incubated *P. falciparum* with boromycin and the highest non-toxic KCl concentration (220 µg/mL). Although a partial antagonistic effect was observed - IC_{50} increased slightly to 2.1 nM - this suggests that potassium ionophores may contribute to, but do not fully explain, boromycin's antiplasmodial activity. $MgCl_2$ was used as a control and, consistent with prior findings, reduced IC_{50} values for both boromycin and chloroquine (208). Pache et al. also reported that other ions (e.g., sodium, ammonium) do not modulate boromycin activity (102). Further investigation of potential targets—including other ion channels such as lithium, ammonium or sodium—is warranted.

In summary, the precise mode of action remains unidentified and should be the focus of future studies.

4.2.4 Boromycin: Activity in different pathogens

Boromycin emerged as one of the most promising compounds in our screen. In addition to its potent antiplasmodial activity, it has demonstrated in vitro efficacy against Gram-positive bacteria (104), *HIV* (105), *Babesia* (107), and *Mycobacteria* (104). A recent study also reported inhibition of intracellular proliferation of *Toxoplasma gondii* and *Cryptosporidium parvum*—both members of the phylum Apicomplexa (106), like *Plasmodium* spp.

Despite this broad-spectrum activity and favorable selectivity, the compound has so far been understudied in the context of *Plasmodium* spp., particularly regarding its in vitro efficacy against human malaria species and its mode of action. This study contributes new insights by demonstrating its potent antiplasmodial activity, while also exploring - but not conclusively identifying - its mode of action.

4.3 Aminoglycosides and nitroimidazoles

Although no antiplasmodial activity was expected based on previous literature, aminoglycosides and nitroimidazoles were included in our screening for completeness, as the compounds were readily available in our laboratory. Consistent with the findings of McColm (140) and Pradines (147), no in vitro activity against *P. falciparum* or *P. knowlesi* was observed.

4.4 Plasmodium knowlesi

4.4.1 Difference of drug activity between Plasmodium falciparum and Plasmodium knowlesi in literature

Although five *Plasmodium* species are known to infect humans and cause malaria, the development and evaluation of novel antimalarials have predominantly focused on *P. falciparum*. A major milestone in expanding research beyond this species was achieved in 2013, when Moon et al. successfully adapted *P. knowlesi* to continuous in vitro culture in human erythrocytes (163). Notably, the other non-*falciparum Plasmodium* species cannot currently be maintained in long-term in vitro culture, which has limited direct comparative studies. *P. knowlesi*, which is phylogenetically more closely related to the non-*falciparum* species than to *P. falciparum*, can therefore serve as a valuable surrogate model. Since the establishment of this culture system, comparative studies have shown that drug susceptibility across human-infecting *Plasmodium* species can vary significantly depending on the compound.

Several studies agree that *P. falciparum* and *P. knowlesi* exhibit similar sensitivity to key antimalarials such as chloroquine, quinine, and dihydroartemisinin (50, 137, 188, 209). However, notable differences in drug susceptibility have also been documented for other compound classes (50, 137, 188, 209). For instance, pyrimethamine, cycloguanil, and trimethoprim have demonstrated greater potency in *P. knowlesi*, whereas blasticidin, DSM1, and mefloquine showed reduced activity compared to *P. falciparum* (50, 188). The most recent findings further emphasize the lower susceptibility of *P. knowlesi* to certain novel antimalarials, including cipargamin and DSM265 (209).

These discrepancies highlight a potential clinical risk: compounds developed exclusively against *P. falciparum* may prove ineffective—or only partially effective—against *P. knowlesi* infections, potentially leading to treatment failure and promoting the emergence of resistance. Regarding antibiotic-based treatments, studies have reported comparable activity in both species for mirincamycin, clindamycin, azithromycin, and doxycycline (50, 137).

4.4.2 Difference of drug activity between Plasmodium falciparum and Plasmodium knowlesi in our study

In this section, we compare the drug susceptibility profiles of *P. falciparum* and *P. knowlesi*, based on our study's findings and the literature, to better understand how these two malaria species respond to a range of therapeutic compounds.

In this study, we analyzed drug susceptibility of *P. knowlesi* after three and six cycles and of *P. falciparum* after 1.5 and three cycles. Drugs that were inactive in *P. falciparum* (oleandomycin, troleandomycin, chlortetracycline, and the aminoglycosides and nitroimidazoles) also showed no activity in *P. knowlesi*. Fast-acting drugs in *P. falciparum* (chloroquine, quinine, chlorotonil A, and ivermectin) exhibited equivalent potency in *P. knowlesi*. The two most active and fast-acting drugs, borrelidin and boromycin, showed slightly higher IC50 values in *P. knowlesi*, but remained in the single-digit nanomolar range, indicating high sensitivity in both species. Moreover, the results for clindamycin and doxycycline after multiple cycles in both parasite species were consistent with the literature (Table 7) (50).

Drugs presenting a delayed death effect in *P. falciparum*, such as demeclocycline, minocycline, eravacycline and josamycin, also presented a delayed death effect in *P. knowlesi*. In contrast, sarecycline, omadacycline, meclocycline, doxycycline and clindamycin, which show a slow onset of action in *P. falciparum*, already showed activity in *P. knowlesi* after three days (three cycles). Therefore, the fold difference between three and six days was insufficient to define a delayed death effect (<15). Nevertheless, these drugs exhibited similar activity in *P. knowlesi* after three days as in *P. falciparum* after six days,

suggesting a late onset of action, though this could not be definitively demonstrated due to assay limitations.

It is important to note that the IC50 values of *P. knowlesi* (A1H1 strain) after three days (three cycles) were smaller than those of *P. falciparum* (3D7 strain) after three days (1.5 cycles). However, for drugs such as meclocycline, sarecycline, omadacycline, doxycycline, and clindamycin, the IC50s of *P. knowlesi* after six days (six cycles) remained higher than those of *P. falciparum* after six days (three cycles). This trend can be summarized as follows:

IC50 3D7 1.5 cycle > IC50 A1H1 3 cycle > IC50 A1H1 6 cycle > IC50 3D7 3 cycle

To fully understand *P. knowlesi* susceptibility to antibiotics, additional studies should implement a 27-hour drug assay for *P. knowlesi*, as done by van Schalkwyk (50, 209), because the delayed death effect in *P. knowlesi* may occur before the first time point of measurement (72 hours), which corresponds to the second or third life cycle. Although we attempted to implement this 27-hour time point, *P. knowlesi* did not show sufficient growth in the negative controls at this time, leading us to exclude these results. Due to the constraints imposed by our measurement schedule, we could not compare the delayed death potency between *P. falciparum* and *P. knowlesi* and thus could not confirm that *P. knowlesi* has reduced delayed death potency for doxycycline, as described by van Schalkwyk (50).

Furthermore, comparisons between drug sensitivity assays for *P. falciparum* and *P. knowlesi* are limited by differences in assay conditions, such as synchronization methods, measurement techniques, and medium composition.

Overall, this study provides a framework for assessing the activity of drugs in various *Plasmodium* species, expanding the characterization of candidate compounds beyond *P. falciparum*. Interestingly, it can be concluded that most antibiotics are equipotent in both *P. knowlesi* and *P. falciparum*, despite the differences in methodology, which limit the predictive accuracy of direct comparisons.

4.5 Advantages and disadvantages of using antibiotics as antimalarials

The repurposing of antibiotics for malaria treatment has gained increasing interest in recent years, prompting a careful evaluation of their potential benefits and limitations in this new therapeutic context

The use of antibiotics as antimalarial agents presents several advantages. Many antibiotics are widely available, cost-effective to produce, and already approved for clinical use, making them suitable for deployment in low-resource settings. Additionally, their established pharmacological profiles and extensive clinical experience can accelerate the repurposing process and reduce the time and cost of clinical development. Antibiotics also provide the potential to treat bacterial coinfections commonly occurring in malaria-endemic regions. Furthermore, antibiotics with antimalarial properties often exhibit different mechanisms of action than those used in current artemisinin-based combination therapies (ACTs), thereby minimizing the risk of cross-resistance. Several agents target the *Plasmodium*-specific apicoplast, an organelle absent in humans, suggesting a favorable selectivity profile. Indeed, antibiotics such as doxycycline (used for malaria prophylaxis), clindamycin (recommended for pregnant women), and sulfadoxine-pyrimethamine (used in intermittent preventive treatment) are already part of malaria treatment protocols (34).

However, despite these advantages, several limitations must be considered. A major concern is the potential for accelerating bacterial resistance through increased antibiotic exposure, particularly in patients with concomitant bacterial infections. Resistance to macrolides and tetracyclines—the two antibiotic classes most active against *Plasmodium* spp. in this study—has already been documented in a broad spectrum of Gram-positive, Gram-negative, and intracellular pathogens (210, 211). This could jeopardize the treatment of common infections such as community-acquired pneumonia (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*), sexually transmitted infections (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*), diarrheal diseases (*Shigella*, *Salmonella*, *Vibrio cholerae*), urinary tract infections (*Escherichia coli*), and sepsis (*Staphylococcus aureus*, *E. coli*, *Streptococcus spp.*), as well as pediatric diseases such as neonatal sepsis (*Streptococcus*

agalactiae) and rheumatic fever (*Streptococcus pyogenes*) (210-217). Of particular concern is the potential resistance development against antibiotics categorized as "essential medicines" by the World Health Organization (WHO), including several drugs shown to have antimalarial activity in this study: clindamycin (e.g., for necrotizing fasciitis, osteomyelitis), doxycycline (e.g., for rickettsial infections, cholera, and STIs), azithromycin (e.g., for yaws, trachoma, and enteric fever), and clarithromycin (e.g., for community-acquired pneumonia and *Helicobacter pylori*) (219).

Conversely, the widespread use of antibiotics for unrelated bacterial infections may also exert unintended selective pressure on asymptomatic *Plasmodium* infections, potentially facilitating the emergence of resistance and thereby compromising their future utility as antimalarial agents.

Boromycin, the most potent compound identified in this study, is not yet in clinical use, and therefore does not currently contribute to antimicrobial resistance. However, as boromycin also exhibits activity against various pathogens, including *Mycobacterium tuberculosis*, HIV, *Toxoplasma gondii*, and *Cryptosporidium parvum*, theoretical concerns about future resistance emergence remain valid if broader clinical use is introduced. Importantly, aside from resistance to folate pathway inhibitors, no widespread resistance to antibiotics has been documented in *Plasmodium* species to date (218).

Evidence from clinical and epidemiological studies further illustrates the complexity of antibiotic use. For example, prophylactic administration of doxycycline for malaria prevention in U.S. military personnel was associated with increased colonization by multidrug-resistant Gram-negative bacteria, although no correlation with Staphylococcus aureus carriage was observed (219, 220).

In addition to concerns about resistance, some antibiotics present practical limitations; for instance, eravacycline requires exclusive intravenous administration, which restricts its use in many settings.

Nevertheless, the use of antibiotics may offer indirect public health benefits. Azithromycin administration in children in sub-Saharan Africa was associated

with a reduction in all-cause childhood mortality (221). While the exact mechanisms remain unclear, a contribution from the compound's antimalarial activity is plausible, given the significant malaria-related mortality in children. Moreover, children with severe malaria frequently present with bacterial sepsis, underscoring the value of simultaneous antibiotic and antimalarial therapy (7, 222).

In conclusion, antibiotics represent promising candidates for antimalarial therapy due to their accessibility, affordability, and dual-action potential against malaria and coinfections. However, their use must be carefully managed to avoid compromising their efficacy in the treatment of bacterial diseases and to mitigate the risk of resistance development in both bacterial and parasitic pathogens.

5 Summary

Introduction

Malaria remains one of the most common diseases and causes of death in the Global South, particularly among children under five years of age. In light of the growing threat of resistance to artemisinin-based combination therapies (ACTs), which currently represent the gold standard in malaria treatment, alternative therapeutic strategies are urgently needed. This study aimed to evaluate the in vitro antiplasmodial activity of selected antibiotics against *Plasmodium falciparum* and *Plasmodium knowlesi*. The latter species, although capable of causing severe disease in humans, has received limited attention in drug research to date. Thus, a central objective of this work was to compare the drug susceptibilities of *P. knowlesi* and *P. falciparum* to support the development of effective treatment options for *P. knowlesi* malaria.

Methods

Parasites were cultured in vitro and exposed to varying concentrations of 20 antibiotics and six reference antimalarials. Drug activity was assessed using the histidine-rich protein 2 (HRP2) enzyme-linked immunosorbent assay for *P. falciparum* and the Sybr Green I assay for *P. knowlesi*. To investigate the role of the apicoplast, drug assays were conducted with supplementation of 200 µM isopentenyl pyrophosphate (IPP). Boromycin, one of the most active compounds identified, was further evaluated for its mode of action using potassium chloride (KCl) supplementation (220 µg/mL) and for cytotoxicity against human HepG2 cells. Stage-specific activity was determined via microscopy at defined time points.

Results

Several tetracyclines (demeclocycline, minocycline, eravacycline, sarecycline, omadacycline, and doxycycline), as well as the macrolide josamycin and the linezolid clindamycin, exhibited a delayed onset of antiplasmodial activity, consistent with the known apicoplast-targeting "delayed death" phenotype. This phenotype was observed in both *P. falciparum* and *P. knowlesi*. In contrast, the macrolide antibiotics borrelidin and boromycin demonstrated rapid parasite

killing, with low nanomolar IC_{50} values in both species (*P. falciparum*: 0.6 nM and 0.9 nM; *P. knowlesi*: 2.7 nM and 6 nM, respectively) after three days. IPP rescue experiments confirmed apicoplast dependence for eravacycline, josamycin, minocycline, and clindamycin, while boromycin and borrelidin retained full activity, indicating a different target. Further analysis showed that boromycin's antiplasmodial activity is not primarily mediated via potassium channel disruption. Its cytotoxicity against human cells was low, with a selectivity index exceeding one million. Notably, boromycin induced pyknotic morphology in all *P. falciparum* blood stages within six hours, indicating rapid and broad-stage parasiticidal activity.

Discussion

The observed drug activity profiles largely align with previously published findings for eravacycline, minocycline, and doxycycline. Chlortetracycline, however, did not demonstrate antiplasmodial activity in our assays, in contrast to earlier reports. We were able to confirm a delayed death effect and apicoplast targeting for josamycin, a macrolide not previously tested in this context.

Boromycin emerged as one of the most potent and selective compounds in our study. Unlike its mechanism in *Mycobacterium* species, its primary mode of action in *Plasmodium* appears to be independent of potassium ionophore activity. Overall, *P. knowlesi* displayed comparable susceptibility to the tested antibiotics as *P. falciparum*, supporting the broader applicability of findings derived from *P. falciparum* models.

Conclusion

Boromycin demonstrated potent, rapid, and stage-independent antiplasmodial activity combined with low human cytotoxicity, making it a promising candidate for further preclinical development. Future work should focus on elucidating its precise mode of action and evaluating its in vivo efficacy.

6 Deutsche Zusammenfassung

Einleitung

Malaria zählt insbesondere bei Kindern unter fünf Jahren zu den häufigsten Erkrankungen und Todesursachen im Globalen Süden. Angesichts der zunehmenden Bedrohung durch Resistenzen gegen die derzeitige Goldstandardtherapie – die Artemisinin-basierte Kombinationstherapie (ACT) – ist die Entwicklung alternativer Therapieoptionen dringend erforderlich. Ziel dieser Arbeit war es, die in-vitro-Aktivität verschiedener Antibiotika gegen *Plasmodium falciparum* und *Plasmodium knowlesi* zu untersuchen. Letztere Spezies wurde bislang in der antimalariellen Wirkstoffforschung weitgehend vernachlässigt. Ein besonderer Fokus lag daher auf dem Vergleich der Arzneimittelsuszeptibilität beider Spezies, um Rückschlüsse auf die Wirksamkeit der getesteten Substanzen auch bei *P. knowlesi*-Malaria ziehen zu können.

Methoden

Zur Bestimmung der antiplasmodialen Wirksamkeit wurden *P. falciparum*- und *P. knowlesi*-Kulturen mit jeweils einem von 20 Antibiotika bzw. sechs Referenzsubstanzen in unterschiedlichen Konzentrationen inkubiert. Das Parasitenwachstum wurde mittels HRP2-ELISA (*P. falciparum*) bzw. Sybr Green I-Assay (*P. knowlesi*) quantifiziert. Zusätzlich wurden IPP-Supplementierungsversuche (200 µM Isopentenylpyrophosphat) durchgeführt, um eine Beteiligung des Apicoplasten als Wirkziel zu überprüfen. Boromycin - eine der potentesten Substanzen - wurde darüber hinaus hinsichtlich seines Wirkmechanismus mittels Kaliumchlorid-Zusatzes und seiner Zytotoxizität an menschlichen HepG2-Zellen weiter charakterisiert. Die Stadienspezifität wurde mikroskopisch zu definierten Zeitpunkten analysiert.

Ergebnisse

Mehrere Tetracycline (Demeclocyclin, Minocyclin, Eravacyclin, Sarecyclin, Omadacyclin, Doxycyclin), das Makrolid Josamycin sowie das Linezolid Clindamycin zeigten, konsistent mit der Literatur, eine verzögerte Wirkung mit Aktivität im Nanomolarbereich erst im zweiten Replikationszyklus von *P. falciparum*. Ein entsprechender Wirkverlauf konnte auch für Demeclocyclin,

Minocyclin, Eravacyclin, Josamycin und Clindamycin bei *P. knowlesi* beobachtet werden. Die beiden Substanzen Borrelidin und Boromycin hingegen zeigten eine rasche parasitenabtötende Wirkung mit IC_{50}-Werten von 0,6 nM bzw. 0,9 nM (*P. falciparum*) und 2,7 nM bzw. 6 nM (*P. knowlesi*) bereits nach 3 Tagen.
Die IPP-Rescue-Experimente bestätigten den Apicoplasten als Zielstruktur für Eravacyclin, Josamycin, Minocyclin und Clindamycin, während Borrelidin und Boromycin unabhängig vom Apicoplasten wirkten. Für Boromycin konnten Kaliumkanäle als primäres Wirkziel ausgeschlossen werden. Die Zytotoxizität an humanen Zellen war sehr gering (Selektivitätsindex > 1.000.000). Zudem führte Boromycin bereits sechs Stunden nach Zugabe in allen Parasitenstadien zu einer pyknotischen Morphologie der Parasiten.

Diskussion

Die Ergebnisse zu Eravacyclin, Minocyclin und Doxycyclin bestätigten frühere Studien. Chlortetracyclin zeigte im Gegensatz zu publizierten Daten keine antiplasmodiale Aktivität. Für Josamycin konnte erstmals ein verzögerter Wirkeintritt sowie eine Apicoplast-Wirkung nachgewiesen werden. Boromycin, eine der wirksamsten Substanzen in dieser Arbeit, wurde aufgrund seines exzellenten Selektivitätsprofils weiter untersucht. Die Wirkweise in *Plasmodium* scheint sich von der in Mykobakterien zu unterscheiden, da Kaliumkanäle nicht als Hauptzielstruktur fungieren. Insgesamt zeigten beide Spezies eine vergleichbare Empfindlichkeit gegenüber den getesteten Antibiotika - ähnlich wie bereits für Mirincamycin, Clindamycin, Azithromycin und Doxycyclin beschrieben.

Schlussfolgerung

Boromycin weist eine hohe, schnell einsetzende Aktivität gegen *Plasmodium* spp. und eine geringe Toxizität für humane Zellen auf. Aufgrund dieser vielversprechenden Eigenschaften sollte es weitergehend auf seinen Wirkmechanismus und seine Wirksamkeit in vivo untersucht werden.

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8 Declaration of own contribution

The practical work of the dissertation was done at the institute of tropical medicine under supervision of Prof. Dr. Benjamin Mordmüller.

The study was designed by Dr. rer. nat. Jana Held in collaboration with PhD Lais Pessanha de Carvalho.

The experiments were performed independently by me, after training by laboratory members PhD Lais Pessanha de Carvalho and Lilith Berner (medical technical assistant). The cytotoxic assay was repeated by PhD Lais Pessanha de Carvalho and the results combined with mine.

The statistical analysis was performed independently by me according to instructions by PhD Lais Pessanha de Carvalho.

I certify that I have written the thesis independently and that I have not used any sources other than those indicated by me.

Publication

Lais Pessanha de Carvalho, Sara Groeger-Otero, Andrea Kreidenweiss, Peter G Kremsner, Benjamin Mordmüller and Jana Held. Boromycin has rapid-onset antibiotic activity against asexual and sexual blood stages of *Plasmodium falciparum* and *Plasmodium knowlesi*. Frontiers in Cellular and Infection Microbiology - Clinical Microbiology. 2021

9 Acknowledgement

At this point I would like to thank all those who made the completion of my doctorate possible:

In the first place I would like to thank Dr. rer. nat. Jana Held for providing me with an extremely interesting topic and a workplace with optimal working conditions.

PhD Lais Pessanha de Carvalho I thank very much for many fruitful suggestions, the companionable atmosphere and being the perfect supervisor.

For the laboratory training and her constant patience and helpfulness I would like to thank Lilith Berner.

I would like to express my thanks to Prof. Dr. Peter Kremsner, director of the Institute of tropical medicine at the University of Tübingen, who has given me the opportunity to carry out this work in his department and to Prof. Dr. Benjamin Mordmüller who has provided himself as my doctoral supervisor.

I would especially like to thank my parents, who have inspired me since a child and awakened my interest in the tropics.