Assessment of the drug activity of anti-infectives against Plasmodium falciparum and Schistosoma mansoni

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1.1. Human parasitic diseases

1.1.1. Epidemiology

Parasitic diseases continue to be a leading cause of morbidity, disability, and mortality in tropical and subtropical regions of the world. A variety of parasites can infect humans, including unicellular protozoans and multicellular metazoan organisms. One of the most devastating parasitic diseases is malaria, with approximately 241 million cases estimated in 2020, most of which occurred in sub-Saharan Africa [1]. In addition, neglected tropical diseases, such as schistosomiasis, African trypanosomiasis, leishmaniasis, Chagas disease, lymphatic filariasis, and onchocerciasis, often affect people in areas of similar geographic distribution as malaria [2]. These diseases are correlated with significant morbidity and often affect people of low socioeconomic status in tropical and subtropical regions of the world. It is estimated that approximately two billion people are affected by neglected tropical diseases, of which, approximately 500 000 people die annually [3]. Schistosomiasis, for instance, is second to malaria among parasitic diseases with the greatest socio-economic impact [4]. Vaccines, medications, vector controls, and other preventive measures can help to control and/or eliminate these diseases.

1.1.2. Prevention, treatment, and control

Maintaining control of parasitic diseases continues to be a challenge, especially in developing countries. Various control measures can be applied to the vectors, infected humans, or the environment to reduce and/or eliminate the pathogens causing the diseases. Vaccinations are one of the most efficient control measures against infectious diseases and are applied to boost the host's immune response to, ultimately, prevent the disease. Billions of people are affected by malaria and schistosomiasis each year without the availability of an effective vaccine. In October of 2021, the RTS,S/AS01 vaccine was approved by the World Health Organization (WHO) for the prevention of *P. falciparum* malaria, recommended for children living in regions with moderate to high transmission [5]. So far, 2.3 million doses have already been administered [1], though the vaccine's

efficacy is low and short-lived and should be used in addition to current malaria control strategies [5,6]. Moreover, insecticide-treated bed nets have been shown to reduce child deaths from all causes by 17% and reduced the incidence of uncomplicated *P. falciparum* malaria by approximately half [7]. Indoor residual spraying with non-pyrethroid-like insecticides is also used, though mixed results have been reported by trials testing its efficacy and impact on malaria incidence rates [8]. Nonetheless, when individuals become infected with a pathogen, detection and rapid treatment with proper chemotherapeutic agents are needed to effectively diagnose and treat patients suffering from the disease. WHO currently recommends six artemisinin-based combination therapies (ACTs) for uncomplicated *P. falciparum* malaria [1]. However, reports of malaria parasites with slow clearance times after treatment with ACTs in Southeast Asia makes treatment, control, and prevention of the spread of possible resistant strains more difficult [9]. Moreover, praziquantel is the treatment of choice for all species of *Schistosoma*, though its efficacy varies, since it primarily targets the adult life cycle stage of the parasite [10]. The development of novel drugs is urgently needed to not only compete with the development of resistance by the malaria parasite to current antimalarials, but to make more effective drugs or drug combinations available to individuals suffering from schistosomiasis.

1.1.3. Malaria

1.1.3.1. About Malaria

Malaria is caused by the protozoan parasite of the genus *Plasmodium* and is a major cause of mortality and morbidity worldwide (Figure 2). An estimated 627 000 malaria deaths were reported in 2020, of which approximately 480 000 were children under the age of 5 years [1]. Malaria is caused by five species of *Plasmodium* parasites including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* through the bite of an *Anopheles* mosquito. *P. knowlesi*, however, is believed to be spread predominantly from primates to humans [11]. *P. falciparum* is by far the most common and virulent of the *Plasmodium* spp. and accounted for 99.7% of all malaria cases in Africa in 2018 [12].

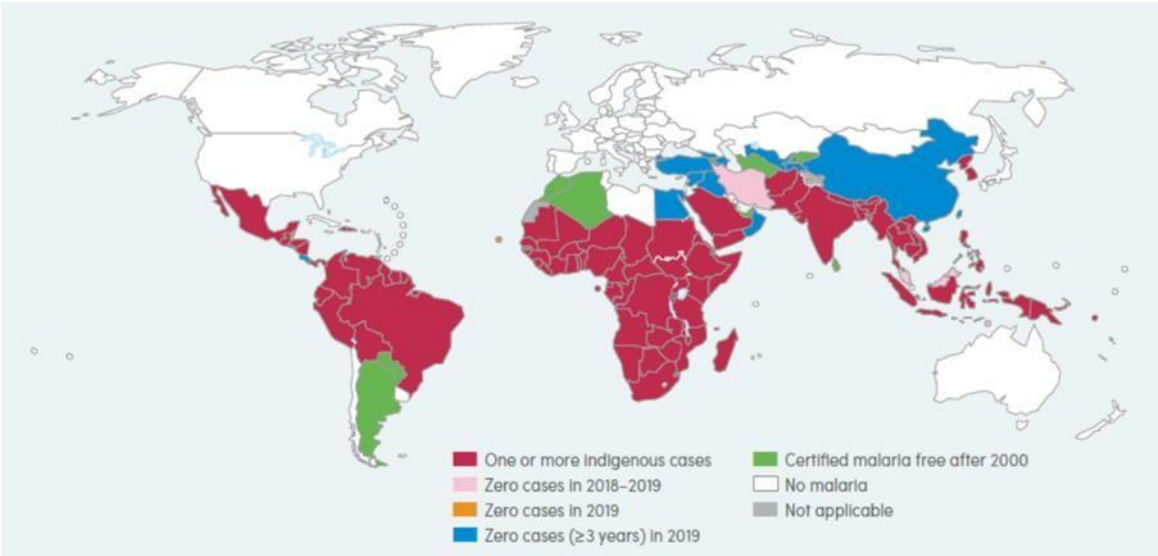

Figure 2: Malaria endemicity worldwide.

Source: Adapted from the World Health Organization – World malaria report 2020

1.1.3.2. Life cycle of *Plasmodium*

The human life cycle of *Plasmodium* spp. begins when an infected female *Anopheles* mosquito feeds on the human host, thereby transferring sporozoites into the subcutaneous tissue along with anticoagulant-containing saliva. The sporozoites travel through the bloodstream to the liver where they infect hepatocytes and mature into schizonts. The asexual stage of the life cycle begins when merozoites are released from the ruptured schizonts and enter the red blood cells. Within one to several days, depending on the species, merozoites further develop into a ring-form stage, continue to the trophozoite stage, and finally to the schizont stage. A small portion of parasites develop into the sexual form, called gametocytes, which are responsible for transmitting the infection during a blood meal to other humans when a female anopheles mosquito feeds on a human containing them. Fertilization takes place between the female and male gametes that emerge from the matured gametocytes inside the gut of the female *Anopheles* mosquito in what is known as the sexual cycle of the parasite. The zygotes become motile ookinetes and invade the midgut wall, where they further develop into oocysts and finally sporozoites, which continue to the salivary glands where they remain until the next blood meal.

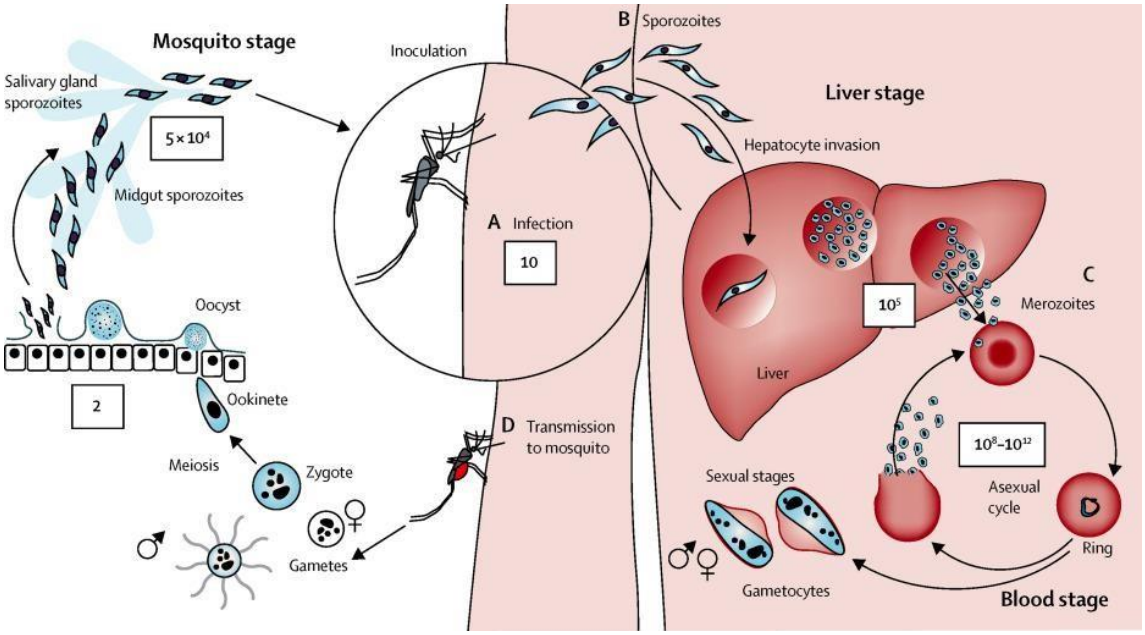

Figure 3: An illustration of the *P. falciparum* life cycle.

Source: White N. et al., Lancet. 2014. (with permission provided by Elsevier - License number: 5243050389760)

1.1.3.3. Symptoms, diagnosis, & treatment of malaria

The disease-causing period of malaria begins with the asexual erythrocytic life cycle. During the parasite's development within the erythrocyte, toxins and waste substances, including erythrocyte membrane products, hemozoin, and glucose phosphate isomerase, are produced and released into the bloodstream when the infected cells lyse, thereby releasing the invasive merozoites [13]. The release of toxins and waste into the bloodstream stimulates macrophages and endothelial cells to secrete cytokines and inflammatory mediators, including tumor necrosis factor, interleukin-1β, interleukin-6, complement factor 5a, and prostaglandin, which results in the malaria-associated symptoms [14]. Symptoms may include fever, fatigue, muscle aches, headaches, and abdominal discomfort, primarily correlated with uncomplicated malaria [15]. Severe malaria complications mainly caused by *P. falciparum* infections, can occur due to acute pulmonary oedemas, acute kidney injuries, and jaundice in adults and severe anemia and cerebral malaria in children [15]. Some individuals, normally adults living in malaria endemic regions who have been exposed frequently to malaria parasite infections in their

lifetime, will have gained some level of immunity, depending on the intensity of transmission, and may be asymptomatic despite being infected with malaria parasites [16]. Microscopy is the gold standard examination technique used when diagnosis is considered for malaria, with a sensitivity of approximately 50-500 parasites/µl [17]. However, rapid diagnostic tests, which are easier to use in the field and have a sensitivity similar to that of microscopy [17], and polymerase chain reaction, though difficult to implement in patient care in endemic areas, but is a highly sensitive diagnostic method, are also utilized. Once malaria has been diagnosed, the patient is treated with an efficacious antimalarial treatment. WHO currently recommends six artemisinin-based combination therapies including artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, dihydroartemisinin-piperaquine, and artesunate-pyronaridine for the treatment of *P. falciparum* infections [1]. ACTs are presently the most important chemotherapeutics in use because they are well-tolerated, act quickly, and are applied in combination to protect them from parasite resistance. However, artemisinin partial resistance emerged, first reported in 2008, and is feared to become a threat to malaria control [18].

1.1.3.4. Antimalarial drug resistance

Antimalarial drug resistance is defined by the ability of the parasite strain to evade killing by a chemotherapeutic drug and continue to propagate in an infected individual, thereby maintaining the presence of the infection [19]. Drug resistance can occur through the selection of parasites harboring genetic mutations that give a selective advantage over sensitive parasites in the presence of drug pressure [20]. Resistance of malaria parasites to various chemotherapeutics often appeared initially in Southeast Asia including to chloroquine (1950s), sulfadoxine-pyrimethamine (1960s), and mefloquine (1980s), which spread swiftly in these regions and eventually appeared in sub-Sahara Africa [19]. However, some studies have found that drug resistance mutations typically carry a fitness cost, thereby giving the wildtype parasite strains a fitness advantage when not exposed to a drug treatment. Similar observations have been made in chloroquine resistant strains, where the prevalence of chloroquine resistant parasites decreased over time, after the removal of chloroquine for the treatment of uncomplicated malaria [21,22]. Nonetheless,

P. falciparum strains resistant to ACTs occurred on the background of preexisting resistance to multiple antimalarial drugs, which include the partner drugs of ACTs [19]. Artemisinin resistance was first observed in 2008 in Cambodia and was characterized by a prolonged *P. falciparum* clearance time following seven days artesunate monotherapy, where a few patients were still parasitemic on day 28 follow-up [18,23]. Artemisinin resistance has continued to spread and emerge independently throughout neighboring countries in the Greater Mekong Subregion [24]. Yet, even with artemisinin resistant malaria parasites in the Greater Mekong Subregion, malaria cases and deaths have been significantly reduced in the past 10 years, resulting in less than 9 000 *P. falciparum* cases and 10 deaths in 2020 [25]. In 2021, the first report of slow clearing malaria parasites after artesunate monotherapy on the African continent came from a longitudinal study conducted in Northern Uganda where 14 of 240 patients who received intravenous artesunate for 24 hours followed by a 3-day ACT treatment course had evidence of artemisinin resistant *P. falciparum* parasites [26]. Even so, antimalarials of the ACT drug class can still cure malaria in malaria endemic regions. However, there is fear that artemisinin resistant strains will spread to or appear *de novo* in sub-Saharan African countries, causing an increase in morbidity and mortality rates. Without effective medicines, treatment and control of malaria would become more challenging. Consequently, there is a need for novel drugs to maintain control of malaria and prevent the continued spread or *de novo* acquisition of resistant strains.

1.1.4. Schistosomiasis

1.1.4.1. About Schistosomiasis

Schistosomiasis, also known as bilharzia, is an infectious parasitic disease caused by flatworms of the genus *Schistosoma*. WHO estimates that approximately 240 million people globally required preventive treatment for schistosomiasis in 2019 [27]. The total number of deaths caused by schistosomiasis each year is difficult to assess, due to the disease's chronic nature, but is estimated to be between 4 400 and 200 000 [27,28]. Schistosomiasis is prevalent in tropical and subtropical areas, especially in African countries where approximately 90% of the cases occur [29], and mostly affects poor and rural communities, including agriculture or fishing communities. There are two major forms

of schistosomiasis, intestinal and urogenital, which are primarily caused by 6 species of *Schistosoma*, including *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi*, *S. guineensis* and *S. intercalatum* [27]. Schistosome infections in humans occur when the skin comes into contact with fresh water contaminated by the infectious stage of the parasite, called cercaria, which are released by the host snail residing in fresh water and penetrate the human skin.

1.1.4.2. Life cycle of *Schistosoma*

The life cycle of *Schistosoma* spp. begins after the cercaria penetrate the human skin and, in the process, shed their forked tail, forming schistosomula. Within 5-7 weeks, the schistosomula travel via the venous circulation to the lungs, heart, and further develop in the liver where the male and female adult worms copulate. The couple migrate to the mesenteric veins of the small and large intestine, though in other species they may also drift to other body organs, including the venous plexus of the bladder as seen in *S. haematobium*, and continue development into adult worms. The female and male adult worms live *in copula* and can produce up to several thousand eggs per day [30]. The eggs are eventually released into the external environment through feces or urine, or become unintentionally lodged in various organ tissues. In fresh water, the eggs form miracidia that can infect the host snail (Figure 4). Once inside the snail, miracidia undergo asexual reproduction and form into sporocysts that continue developing into cercariae, thereby continuing the life cycle after infecting the new human host.

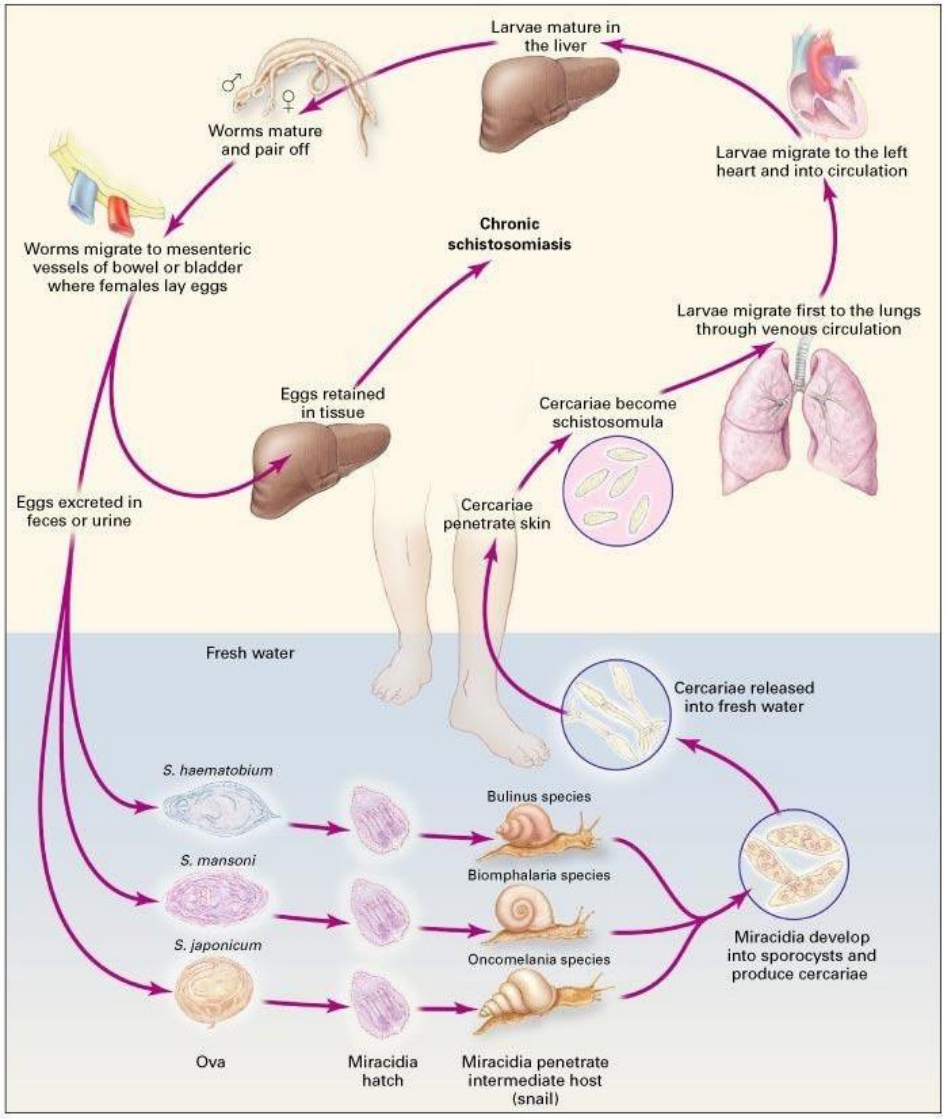

Figure 4: An illustration of the life cycle of Schistosoma [31].

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1.1.4.3. Symptoms, diagnosis, & treatment of schistosomiasis

Once the pair of adult worms have developed within the human body and areproducing eggs, some eggs become trapped in the host's tissues, including the liver, intestines, and spleen, thereby inducing an immune response, severe inflammation, and

granuloma formation [31]. Typical symptoms in intestinal schistosomiasis include abdominal pain, diarrhea, and blood in the stool [32]. Chronic and repeat infections can result in irreversible damage to body organs, such as the bladder, after long-infections with *S. haematobium*, which may lead to bladder cancer and cervical cancer [33]. Schistosomiasis is either diagnosed through the detection of parasite eggs in the stool or urine by microscopy, or by various antigen detecting methods. Praziquantel is the only recommended drug for the treatment of schistosomiasis and is active against all *Schistosoma* spp. [34]. A standard treatment of praziquantel is done with a single oral dose of 40 mg/kg body weight for *S. mansoni, S. haematobium,* and *S. intercalatum* infections, but may also be given at a single dose of 60 mg/kg for *S. japonicum* and *S. mekongi* infections [32]. It is a well-tolerated, effective, and low-cost drug, but is solely active against the adult life cycle stage. Schistosomes in the prepatent period are not affected by praziquantel, which is a limit to schistosomiasis control and elimination efforts.

1.1.4.4. Reduced drug sensitivity in schistosomes

A major issue in schistosomiasis prevention, treatment, and control is that praziquantel is the only readily available drug. Resistance of *Schistosoma* parasites to praziquantel has not been confirmed, but individual cases of treatment failure to praziquantel over the past decades [35] and the successful development of resistant strains in the laboratory, based on *S. mansoni* transient receptor potential melastatin ion channel mutations [36], has raised concerns in regard to its existence. Moreover, clinical schistosome isolates with reduced sensitivity to praziquantel have been isolated after mass drug administration programs in Egypt and Senegal [37–39]. So far, it is unclear as to whether or not resistant strains truly exist, however, data on less susceptible *S. mansoni* strains to praziquantel are occasionally reported [18–20]. Moreover, a cure rate by praziquantel of 100% has rarely been achieved, which may be explained, among other things, by praziquantel's lacking drug activity against prepatent stages of the parasite's life cycle. The need for new antischistosomal compounds optimally exhibiting broad activity against all stages of the parasite's life cycle in humans is essential to maintain control and effectively treat patients suffering from the disease.

1.2. Drug development

1.2.1. Approaches in drug development

Drug development starts with the identification of drug candidates that are progressively developed from preclinical development to clinical development until drug approval, if successful. Approaches to identify drug candidates are diverse and range from target-based approaches to whole parasite testing. Phenotypic screenings allow for the testing of a substance's activity towards the whole parasite and inform on the accessibility and inhibitory effects early on. In contrast, in target-based approaches a substance is tested for its inhibitory effect against a predefined parasitic molecule that ideally represents a target/molecular pathway that is essential to parasite survival. Traditional drug discovery approaches include the *de novo* identification of novel compounds, which can be highly time-consuming, costly, and have a high risk of failure. Drug repurposing is a time and cost-effective strategy to identify new therapeutic purposes of drugs already approved for human use. Drug repurposing strategies also allow researchers to use already available clinical safety, toxicological, and pharmacokinetics data. Repurposed candidates can be tested straight forward in humans in clinical trials to test their efficacy against the new target disease. For example, antibiotics of the tetracycline class, were first used to treat patients with various bacterial infections, but were later on used and continually developed for the treatment of malaria [40]. Similarly, antimalarials can be repurposed for use against other infectious diseases or in cases of co-infections, including with schistosomiasis.

1.2.2. Antimalarials in development

Antimalarial drug resistance is a major obstacle in the elimination of malaria and documented cases of resistant strains have occurred for almost all antimalarial drugs currently in use, although ACTs are still efficacious in the treatment of uncomplicated malaria. Typically, antimalarial drugs are given in combination with other drugs, including the ACTs, to protect them from parasite resistance [19]. Beginning in the early 1980s, artemisinins were given as a monotherapy and were shown to be safe and highly efficacious, but with a high recrudescence rate, predominantly attributed to nonadherence

to the lengthy regimen [41]. Consequently, artemisinins are now given in combination with long-acting partner drugs that clear residual parasites to ensure an efficacious treatment of uncomplicated malaria. Until new antimalarial compounds become available, one strategy is to further combine an ACT with an additional partner drug, thereby resulting in triple or even quadruple artemisinin-based combination therapies [19]. So far, dihydroartemisinin-piperaquine plus mefloquine and artemether-lumefantrine plus amodiaquine have been shown to be efficacious, well-tolerated, and safe [42]. Artesunate-pyronaridine combined with a non-ACT is also currently being assessed in two clinical trials (NCT03726593, PACTR202010540737215) in adults in the Greater Mekong Subregion and in children in sub-Saharan Africa. Nonetheless, the identification of antimalarial agents with novel modes of action is the ideal approach to combat the emergence of resistance to existing drugs. New compounds with a different antiplasmodial mechanism are currently in the late stages of development for uncomplicated falciparum malaria, including ganaplacide, an imidazolpiperazine, cipargamin, a spiroindolone, and artefenomel, a second-generation trioxolane [43].

1.2.3. Antischistosomal compounds in development

Praziquantel is currently the only drug available for the treatment and control of all *Schistosoma* spp. This mono chemotherapeutic strategy for schistosomiasis, however, presents with several challenges including that it is not active against juvenile worms and the cure rate is usually between 60-90% in endemic countries [44]. A drug or drug combinations targeting the adult and prepatent life cycle stages of *Schistosoma* would be more effective in treating patients and would significantly reduce pathogen transmission rates. Treatment strategies currently in development include *de novo* drug development, combining praziquantel with a partner drug, and/or the repurposing of drugs indicated for other diseases.

Beginning in the 1980s, attempts were made to partner praziquantel with oxamniquine, a semisynthetic tetrahydroquinoline, for the treatment of *S. mansoni* infections [45]. Oxamniquine, however, is primarily only active against *S. mansoni* and reports of resistant strains have limited the use of this drug [46]. Moreover, studies investigating the *in vitro* drug activity of the antimalarial compounds artesunate [47], mefloquine [48], primaquine

[49], and pyronaridine [50] found them to be moderately to highly active against schistosomula *in vitro,* though other compounds such as artemether [51], chloroquine [52], ferroquine [52], and quinine [52] were minimally active or not active. *In vivo* experiments testing the efficacy of artemether (given once a day for three days at 300 mg/kg) [53], artesunate (given once a day for three days at 300 mg/kg) [53], and mefloquine (given once at 400mg/kg) [54] against juvenile stages in mice revealed a high worm burden reduction of 97%, 77%, and 98%, respectively. Antimalarial drugs tested against adult worms *ex vivo* were either inactive or showed minimal drug activity, though mefloquine and amodiaquine have been reported as active at 11µM [48]. *In vivo,* using the mouse model, mefloquine given once at 400mg/kg [54] and artesunate given once for two days at 400mg/kg, reduced the adult worm burden by 77% and 33%, respectively.

Clinical studies investigating the prophylactic effects of artemether and artesunate reported varying efficacies (%) against *Schistosoma* parasites: Artesunate against *S. japonicum* (100%) [55], artemether against *S. japonicum* (60-100%) [56–60], artemether against *S. mansoni* (50%) [61], and artemether against *S. haematobium* (25%) [62]. Moreover, clinical trials testing the antischistosomal activity of antimalarial compounds as a monotherapy or in combination with other antimalarials or praziquantel reported the following cure rates (%) against *Schistosoma* parasites: Artesunate monotherapy against *S. mansoni* (31%) [63] and *S. haematobium* (25-65%) [64,65]; mefloquine monotherapy against *S. mansoni* (no effect) [64] and *S. haematobium* (21%) [64]; artesunate – mefloquine against *S. haematobium* (61%) [64]; artesunate – praziquantel against *S. haematobium* (81%) [65]; mefloquine – praziquantel against *S. haematobium* (26%) [66]; artesunate – mefloquine – praziquantel against *S. haematobium* (29%) [66]; artesunate – sulfadoxine – pyrimethamine against *S. mansoni (59-100%)* [67,68] and *S. haematobium* (100%) [69]*;* artesunate – amodiaquine against *S. haematobium* (87%) [69]; artemether – praziquantel against *S. mansoni* (87%) [70]. Most clinical trials, however, are difficult to draw conclusions from since they vary in design, have a low sample size, or no significant difference was observed from the current standard of treatment praziquantel monotherapy. Moreover, a major drawback in regard to the use of antimalarials in combination with praziquantel is the fear of the contribution to the emergence of drug-resistant malaria parasites.

Although it is quite unlikely that antimalarial drugs with a proven antischistosomal efficacy would be recommended for the treatment of schistosomiasis, such compounds may either be used as a starting point for further chemical development towards specificity for *Schistosoma* or used for schistosomiasis in case the drugs become abandoned for malaria treatment and are of great interest in regions where co-infections commonly occur. Thus, there is further need to evaluate compounds for their uses as antischistosomal agents, especially compounds active against the juvenile and adult life cycle stages.

1.2.4. Drugs for coinfections with *Plasmodium* and *Schistosoma* parasites

Many people in sub-Saharan Africa, who are affected by malaria, may also be suffering from other neglected tropical diseases, including schistosomiasis, since both parasites – *Plasmodia* spp. and *Schistosoma* spp. - have a large geographical overlap in endemicity [71]. This makes finding a drug used for both diseases more appealing, since co-infection rates can reach even greater than 50% in some areas [72–74]. An implementation scenario of such a dual use drug could be mass treatment campaigns, where populations in endemic areas get regularly treated irrespective of their infection status. Remarkably, *Plasmodium* and *Schistosoma* parasites both digest hemoglobin, thereby releasing high quantities of toxic free heme, but use intracellular processes to convert it into hemozoin, a less toxic form [75,76]. Several antimalarial compounds, including quinolines, inhibit hemozoin formation, thereby killing the malaria parasite [77]. Existing antimalarials or newly developed antiplasmodial compounds may be of interest to not only target the prepatent stages of *Schistosoma*, but to be used against both parasites in concomitant infections. Approved antiplasmodial compounds may be further used for the development of new interventions against the *Schistosoma* parasites, either as antimalarial treatments with an add-on effect against schistosomes or as a starting point for schistosomiasis drug development [78].

1.3. Potential drug candidates

1.3.3. Antibiotics

Antibiotics are an attractive group of antimicrobial compounds known to be active not only against bacteria, but also protozoa, including the malaria parasites. Tetracyclines were one of the first antibiotics used for the treatment of uncomplicated *P. falciparum* malaria. Many antibiotics of the tetracycline class exist today, including doxycycline, minocycline, tigecycline, and the newer compound eravacycline [79]. Doxycycline is the most widely employed tetracycline and has been used for prophylaxis against malaria and in combination with artesunate or with quinine as a follow-up treatment for severe malaria [80]. In *Plasmodium* spp*.,* antibiotics, including the tetracyclines, are known to target the apicoplast, a plastid-like organelle of endosymbiotic ancestry [40]. The apicoplast's major function is isoprenoid precursor biosynthesis, which is essential for parasite survival [81]. Thus, new antibiotics of existing drug classes may be active against the malaria parasite, by potentially targeting the apicoplast, but not human cells, therefore making it an ideal drug target [79]. The antibiotic doxycycline, for example, is used for malaria prophylaxis and is combined with other antimalarials to treat bacterial co-infections [80]. Additionally, eravacycline, a fully synthetic novel tetracycline developed to overcome the classical resistance mechanisms of bacteria against tetracyclines, gained fast-track designation by the Federal Drug Administration for complicated intra-abdominal infections [82]. Eravacycline has broad-spectrum activity against Gram-positive and Gram-negative bacteria, and other multidrug-resistant microorganisms [82]. Eravacycline could be investigated for its antiplasmodial activity and evaluated as a treatment option for malaria patients with bacterial co-infections. Furthermore, only a few publications mention the drug activity of antibiotics, including the tetracyclines [83], against the *Schistosoma* parasite, though most are shown to be inactive [78]. Nonetheless, antibiotics may be used to target other organelles, including the mitochondrion, or important intracellular mechanisms within the *Schistosoma* parasite [84].

1.3.4. Epigenetic regulators

Another attractive class of malaria drug candidates are the epigenetic regulators histone deacetylase inhibitors (HDACi). Histone deacetylase enzymes (HDACs) play an essential role in the wrapping and unwrapping of DNA around histone octamers by removing acetyl groups from the side chain of certain lysine residues [85]. Inhibition of HDACs obstructs important cellular processes, including replication, DNA repair, and transcriptional and post-translational mechanisms [86]. So far, most HDACi are being developed to treat certain cancers and many, including vorinostat, belinostat, and panobinostat, are already FDA approved [87]. Current data indicate HDACi as attractive drug candidates against parasitic diseases caused by *Plasmodium, Schistosoma, Leishmania, Trypanosoma,* and *Toxoplasma* [88]. Apicidin was one of the first HDACi tested against *P. falciparum* and exhibited broad-spectrum antiprotozoal activity *in vitro* and *in vivo* in mice [89]. HDACi are shown to be active against multiple life cycle-stages of *P. falciparum*, but their use is limited due to concomitant toxicity to human cells, especially pan-HDAC inhibitors targeting multiple HDACs [90]. So far, up to six HDACs in *P. falciparum* have been identified, which share only a certain degree of sequence identity with human HDACs. One idea is to develop more selective inhibitors, including to plasmodial class II homologues, such as hHDCA6, which also target non-histone proteins (alpha-tubulin, Hsp90) [91,92]. Similarly, HDACs are also present in the *Schistosoma* parasite, including class I HDACs, such as smHDAC1, smHDAC3, and smHDAC8, and are being investigated as potential drug targets [93]. For these reasons, HDACi should be further investigated for their antiplasmodial activity and expanded to include other parasitic diseases, such as schistosomiasis.

1.3.5. Compounds targeting the hemozoin synthesis pathway

Schistosomes and malaria parasites digest blood/hemoglobin, which causes toxic free heme to be released. Both parasites have developed intracellular detoxification mechanisms to rapidly convert heme into a less toxic crystallized form called hemozoin. Drugs inhibiting this pathway can lead to the formation of oxygen species within the cell, thereby causing oxidative stress, which ultimately leads to the parasite's death [94]. Several antimalarial classes including the quinolines, benzo-naphthyridines, azoles,

isonitriles, xanthones, and phenothiazines are known to increase intracellular free heme by inhibiting hemozoin formation [94,95]. For example, chloroquine, a quinoline derivative, interacts with the μ-oxo dimer of oxidized heme and prevents hemozoin formation [96]. Similarly, pyronaridine, a benzo-naphthyridine and methylene blue, a tricyclic phenothiazine, inhibit ß-hematin formation [95,97]. Thus, novel drugs targeting both parasites can be identified or developed *de novo* to potentially target the hemozoin synthesis pathway.

1.4. Scope and specific objectives:

Parasites are responsible for causing a number of harmful diseases in humans, especially in tropical and sub-tropical regions of the world. Malaria, an acute and life-threatening disease, causing significant mortality, and schistosomiasis, a predominantly chronic disease leading to long-term morbidity, which can lead to the development of bladder cancer, as seen in *S. haematobium* cases, are often found together in the same geographical area [72–74]. The development of vaccines and novel drugs, preferably with a new mode of action, are needed to decrease mortality associated with these diseases. ACTs, are currently recommended by WHO for the treatment of patients with uncomplicated malaria, but recent reports from Southeast Asia show some *P. falciparum* strains to be cleared more slowly by artemisinins [18,23]. There is concern that resistance to artemisinin and its derivatives may spread to sub-Saharan Africa from Southeast Asia or the *de novo* acquisition of resistance may occur in sub-Saharan Africa, the most affected area by malaria. In 2021, the first cases of artemisinin resistant parasites, based on mutations in the *kelch13* gene, were reported in Uganda [26]. New medications are therefore needed to control the spread and development of resistance by the parasites to currently used drugs. Moreover, drugs can be repurposed or used as novel drug candidates to be further developed to be used against neglected tropical diseases. Schistosomiasis, a disease caused by parasitic flatworms, is the second most common socio-economically devastating tropical parasitic disease next to malaria [32]. Praziquantel, the only effective drug currently in use, mainly targets the adult life cycle stage of the parasite, and the cure rate rarely reaches 100%, which makes disease control more difficult.

The aim of this thesis was to assess the activity of compounds targeting organelles of prokaryotic descent, epigenetic regulators, and the hemozoin synthesis pathway in *Plasmodium* and *Schistosoma* parasites. This work aims to prepare for future drug trials in *Plasmodium* and *Schistosoma*-coinfected individuals in endemic regions to assess a drug´s effect on the concomitant parasites.

In the first study, several antibiotics were carefully selected, based on already published data from preclinical and clinical studies, and evaluated their drug activity against *P. falciparum* and *S. mansoni*. Antibiotics act against bacteria, including organelles in eukaryotes of prokaryotic descent, by inhibiting vital cellular processes such as the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins. Therefore, the apicoplast of *P. falciparum* was evaluated as a potential drug target.

In the second study, the *in vitro* potency of novel histone deacetylase inhibitors (HDAC6-inhibitors) was evaluated against *P. falciparum* clinical isolates collected in Lambaréné, Gabon and surrounding areas. Selective inhibitors of human HDAC6 (hHDAC6), a class II enzyme, express reduced levels of cytotoxicity to human cells compared to HDAC class I inhibitors by targeting non-histone proteins and class II homologues that are also present in *P. falciparum*. HDAC homologues in the *Schistosoma* parasite have also been identified and would make for interesting targets for novel drugs.

In the final study, antiplasmodial compounds targeting the hemozoin synthesis pathway were evaluated, especially pyronaridine and methylene blue, for alternative treatment strategies of *Schistosoma* infections, especially for the juvenile stages.

2. Results and discussion

2.1 Chapter 1: Tetracyclines against *Plasmodium falciparum.*

***In vitro* activity of eravacycline, a novel synthetic halogenated tetracycline, against the malaria parasite Plasmodium falciparum.**

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In vitro activity of eravacycline, a novel synthetic halogenated tetracycline, against the malaria parasite *Plasmodium falciparum*

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ABSTRACT

Objectives: Eravacycline is a novel synthetic halogenated tetracycline derivative with a broad antibacterial spectrum. Antibiotics, including tetracyclines, have been used for prophylaxis and, more rarely, for the treatment of malaria for several decades. The rise in drug-resistant malaria parasites renders the search for new treatment candidates urgent. We determined the in vitro potency of eravacycline against *Plasmodium falciparum* and investigated the apicoplast as a potential drug target.
Methods: Four tetracyclines, including eravacycline, tetracycline, tigecycline, and doxycycline, and the lincosamide clindamycin, were tested in 3-day and 6-day in vitro susceptibility assays of *P. falciparum* laboratory strain 3D7 and/or of clinical isolates obtained from 33 *P. falciparum* infected individuals from Gabon in 2018. Assays with isopentenyl pyrophosphate substitution were performed to investigate whether apicoplast-encoded isoprenoid biosynthesis is inhibited by these antibiotics.
Results: Eravacycline showed the highest activity of all tetracyclines tested in clinical isolates in the 3-day and 6-day assays. Substitution of isopentenyl pyrophosphate in vitro using the laboratory strain 3D7 reversed the activity of eravacycline and comparator antibiotics, indicating the apicoplast to be the main target organelle.
Conclusions: These results demonstrate the potential of novel antibiotics, and eravacycline, as candidate antimalarial therapies.

1. Introduction

Malaria is a life-threatening disease caused by protozoan parasites of the genus *Plasmodium*. Six species of *Plasmodium* are described to infect humans, of which *Plasmodium falciparum* is the deadliest. The World Health Organization (WHO) reported a total of 228 million malaria cases and 405 000 deaths in 2018 [1]. Although artemisinin-based combination therapies (ACTs), the current gold standard, are highly effective malaria treatments, reduced sensitivities evidenced by the slow clearing of *P. falciparum* parasites have been reported in Southeast Asia [2]. In the Greater Mekong Region, ACT failures are increasingly reported and reached 50% when dihydroartemisinin-piperaquine was recently tested [3]. A major concern is the emergence and spread of multidrug-resistant strains in Sub-Saharan Africa, where approximately 90% of all *P. falciparum* malaria cases occur [1]. Antimalarial candidates with a new mode of action are scarce, but are urgently needed for the development of prospective malaria medicines.

Antibiotics are an interesting group of anti-infectives with some known to be active not only against bacteria, but also against protozoa, including the malaria parasites. Tetracyclines are broad-spectrum antibiotics used to treat a number of bacterial diseases including rickettsial diseases, borreliosis (Lyme's disease), and chlamydial infections [4–6]. They were among the first antibiotics

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to be used for treatment of uncomplicated *P. falciparum* malaria since the 1950s [7]. Emergence of chloroquine resistance led to further investigation of tetracyclines, especially doxycycline, which was recommended by the WHO for *P. falciparum* malaria chemoprophylaxis in 1985 and used particularly by travellers from the USA [7,8]. In combination with artesunate or with quinine, doxycycline can be used as follow-up treatment for severe malaria or as an alternative combination treatment for uncomplicated malaria when a standard ACT is not available [9].

Antibiotics act against bacteria, including organelles in eukaryotes of prokaryotic descent, by inhibiting important cellular processes such as the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins [10,11]. In *Plasmodium spp.*, many antibiotics, including tetracyclines and lincosamides, target the apicoplast, which has the essential function of isoprenoid precursor biosynthesis during blood-stage development [12,13]. The apicoplast is a plastid-like organelle of endosymbiotic ancestry and is an attractive drug target as it is absent in humans and essential for parasite survival [14]. Antibiotics acting against the apicoplast of *Plasmodium* parasites cause a typical 'delayed death' effect; following exposure, parasites continue to replicate and invade new erythrocytes, where they die, even in the absence of the drug [14].

Eravacycline (TP-434) is a fully synthetic novel tetracycline containing a fluorine group at C7 and a pyrrolidinoacetamido group at C9, developed to overcome the classical resistance mechanisms of bacteria against tetracyclines. Like other tetracyclines, it inhibits bacterial translation by binding to the 30S ribosomal subunit [15]. Eravacycline is related to tigecycline, another novel tetracycline derivative containing an *N,N,*-dimethylglycylamido group, that was developed recently [16]. Eravacycline has broad-spectrum activity similar to or broader than that of tigecycline, against Gram-positive and Gram-negative bacteria, and multidrug-resistant microorganisms [17]. It received Food and Drug Administration (FDA) fast-track designation for complicated intra-abdominal infections [18]. Tigecycline is also approved for the treatment of complicated intra-abdominal infections and preclinical studies showed antiplasmodial activity superior to other tetracyclines [19].

We tested eravacycline for its in vitro potency against *P. falciparum* and compared it against antibiotics approved for use in humans with reported antiplasmodial activity. Furthermore, initial studies to shed light on the mode of action were performed.

2. Materials and methods

2.1. Plasmodium falciparum culture

Plasmodium falciparum laboratory strain 3D7 (chloroquine-sensitive) blood stages were maintained in continuous in vitro culture as previously described [20]. Parasites were kept in complete culture medium (RPMI 1640, 25 mM 4-(2-hydroxyethyl) piperazine-N'-(4-butanesulphonic acid), 2 mM L-glutamine, 50 μg/mL gentamicin, and 0.5% w/v albumax) at 2.5% haematocrit and incubated at 37 °C in an incubator at 5% CO_2 and O_2 with daily change of medium. Synchronization was performed with 5% sorbitol twice a week [21].

2.2. Sampling of P. falciparum clinical isolates

Participants with uncomplicated malaria residing in Lambaréné and surrounding villages in Gabon were enrolled between January and June of 2018. Informed consent was obtained from the study participants or their legal representative, if minor. Inclusion criteria included written informed consent, ages either 1–5 years (paediatric cohort) or ≥18 years (adult cohort), and *P. falciparum* monoinfection with a parasitaemia above 1000 parasites/μL as assessed by Giemsa-stained thick blood smear [22]. To obtain clinical *P. falciparum* isolates, a venous blood sample was taken in a lithium heparin/EDTA tube (Monovette) and processed in the in vitro growth inhibition assay within 9 h in a candle jar.

All methods were performed in accordance with relevant guidelines and regulations.

2.3. Compounds

Doxycycline hyclate (Sigma-Aldrich) (molecular weight [MW]: 513), clindamycin hydrochloride (Sigma-Aldrich) (MW: 461), tigecycline hydrochloride (Hycultec) (MW: 586), and tetracycline hydrochloride (Sigma-Aldrich) (MW: 444) served as controls and were dissolved in dimethyl sulphoxide (DMSO) at 100 mM; eravacycline (Hycultec) (MW: 559) was dissolved in DMSO at 50 mM. All stocks were freshly prepared and stored at −20 °C. Maximum concentration of solvent DMSO in the in vitro growth inhibition assay was 0.4% and did not interfere with parasite growth in pilot experiments.

2.4. In vitro P. Falciparum growth inhibition assay

Growth inhibition assays were performed according to standard procedures [23]. Ninety-six well-plates were pre-dosed with a three-fold serial dilution of the respective drug in complete culture medium with the following range of concentrations: chloroquine 1.0–1000 nM; clindamycin 1.7–50 000 nM; doxycycline 3.4–250 000 nM; eravacycline 0.3–30 000 nM; tetracycline 1.2–100 000 nM; or tigecycline 1.0–2 000 000 nM. Ring-stage parasites of 3D7 and clinical *P. falciparum* isolates were adjusted to a parasitaemia of 0.05% with O^+ erythrocytes and the haematocrit was set to 1.5%.

For the 3D7 laboratory strain, three different assay conditions were performed: 1) incubation time of 3 days (standard condition) 2) incubation time of 6 days (to investigate the delayed death effect), and 3) incubation time of 6 days in complete medium supplemented with 200 μM isopentenyl pyrophosphate trilithium salt (IPP; Sigma-Aldrich) to investigate target (apicoplast) specificity [24]. For clinical isolates, only the two different incubation times were investigated: 3 and 6 days. After 3 days or 6 days of incubation at 37 °C, plates were freeze-thawed three times and analysed by measurement of *P. falciparum* histidine-rich protein 2 (HRP2) with an enzyme-linked immunosorbent assay (ELISA) [25]. Only growth inhibition assays with successfully grown and propagating parasites reflected by a two-fold increase of absorbance between complete and no inhibition within the 6 days were included in the 50% inhibitory concentration (IC_{50}) analysis (quality criteria) and compared to clindamycin and doxycycline as the positive controls. Compounds tested in the 3-day growth inhibition assays where parasite propagation did not reflect a two-fold increase were considered to be inactive and the IC_{50} was labelled with 'greater than max concentration' tested. All experiments were performed in duplicate technical replicates.

2.5. Statistics

Individual 50% inhibitory concentrations were determined by non-linear regression analysis of log-concentration-response curves using the drc-package v3.0–1 of R version 3.4.2 [26]. Data for the clinical isolates were presented using the median IC_{50} and the interquartile range (IQR). IC_{50} values calculated to be over the highest tested drug concentration were substituted with the highest tested concentration value if parasites were dead at the highest tested drug concentration (indicated by a low absorption value of confirmed dead parasites from the same participant).

3. Results

Eravacycline and comparator antibiotics tetracycline, doxycycline, tigecycline, and clindamycin were tested for their in vitro potency against *P. falciparum* laboratory strain 3D7 (Fig. 1). The potency against the parasites was evaluated simultaneously after 3 days as well as after 6 days, in in vitro drug exposure assays. In contrast to the 3-day assay, where most compounds had an IC_{50} in the micromolar range and, thus, were barely active at concentrations that can be reached in vivo, all tetracyclines were highly active after 6 days exposure with eravacycline being the most potent derivative with an IC_{50} in the low nanomolar range (Table 1). The lincosamide clindamycin was not active after 3 days, but exhibited the lowest IC_{50} (6 nM) in the 6-day assay.

Eravacycline, similar to the comparator antibiotics, resulted in an increase in antiplasmodial activity in a prolonged incubation time over 6 days (approximately three asexual cycles) (Table 1). The 'delayed death' phenomenon of *P. falciparum* following antibiotic activity is known to result from loss of the apicoplast in progeny cells [27]. To validate apicoplast encoded pathways as the target of antibiotic activity, parasite culture medium of 6-day drug susceptibility assays was supplemented with isopentenyl pyrophosphate to rescue parasite viability. IC_{50}s of eravacycline and comparator antibiotics after 6 days with IPP supplementation were nearly as high as those of the 3-day assays, suggesting the apicoplast is an essential target organelle (Table 1). Only few outliers were present in the data for most antibiotics tested and only a slight difference was observed in the 6-day assays between 3D7 and the clinical isolates.

As a next step, eravacycline and comparator drugs were tested in genetically diverse *P. falciparum* isolates [28] collected from 46 infected individuals living in Gabon in an area of high malaria prevalence [29]. Median age (Q1–Q3) of all participants was 5 years (3.5–22 years) and median parasitaemia (Q1–Q3) of all participants assessed by thick blood smear was 6726 parasites/μL (2223–25 859). Of the 46 clinical isolates subjected to in vitro drug susceptibility testing, 33 parasite isolates were successfully grown in vitro and fulfilled quality criteria for IC_{50} analysis. Among the tetracyclines, eravacycline was the most active compound after 6 days as well as after 3 days exposure with an IC_{50} of 29 nM and 69 nM, respectively (Table 2). High potency of clindamycin against parasites circulating in malaria endemic Gabon was confirmed in vitro.

Table 1
Activity of antibiotics against the *Plasmodium falciparum* laboratory strain 3D7.

Compounds	3-day assay IC_{50} (SD) in nM	6-day assay IC_{50} (SD) in nM	6-day assay + IPP IC_{50} (SD) in nM
Eravacycline	1996 (252)	14 (8)	599 (252)
Tetracycline	56 436 (25,003)	340 (156)	20 654 (15 593)
Doxycycline	9684 (2109)	241 (87)	5926 (2617)
Tigecycline	5728 (1881)	38 (25)	2413 (197)
Clindamycin	>100 000	6 (2)	3338 (557)

Antibiotics were tested in 3-day, 6-day, and a 6-day plus isopentenyl pyrophosphate (IPP) supplementation in vitro drug susceptibility assays. Mean 50% inhibitory concentrations (IC_{50}) and standard deviations (SD) in nM are shown. Each experiment was performed at least three times.

Table 2
Activity of antibiotics against *Plasmodium falciparum* clinical isolates from Gabon.

Compounds	3-day IC_{50} (IQR) in nM	6-day IC_{50} (IQR) in nM
Eravacycline	69 (35–142)	29 (13–157)
Tetracycline	2536 (1421–4127)	860 (543–2405)
Doxycycline	4523 (1743–77 260)	881 (399–2652)
Clindamycin	31 (15–66)	8 (4–34)

Antibiotics were tested in a 3-day and a 6-day in vitro drug susceptibility assay. Median IC_{50} and interquartile range (IQR) in nM of antibiotics tested against 33 clinical isolates are shown. Each experiment was performed in duplicate and done once per isolate.

4. Discussion

This study demonstrated high activity of the novel tetracycline derivative eravacycline against the culture-adapted *P. falciparum* strain 3D7 and clinical isolates in the 6-day assay, with similar IC_{50}s in the low nanomolar range. It was almost three times more active than tigecycline and about 17 times more active than doxycycline in the context of prolonged drug exposure (6-day assay) in the 3D7 laboratory strain. As expected, none of the antibiotics inhibited propagation of the laboratory strain parasites within the first cycle (3-day assay), although there was still a measurable inhibition exerted by most compounds, with eravacycline having the highest activity (1996 nM). This could indicate additional off-target activity of eravacycline at biologically relevant concentrations, as a mean peak plasma concentration of 3.8 μM is reached when administered at the standard dose of 1 mg/kg [30]. Interestingly, the delayed death effect for eravacycline and clindamycin was less

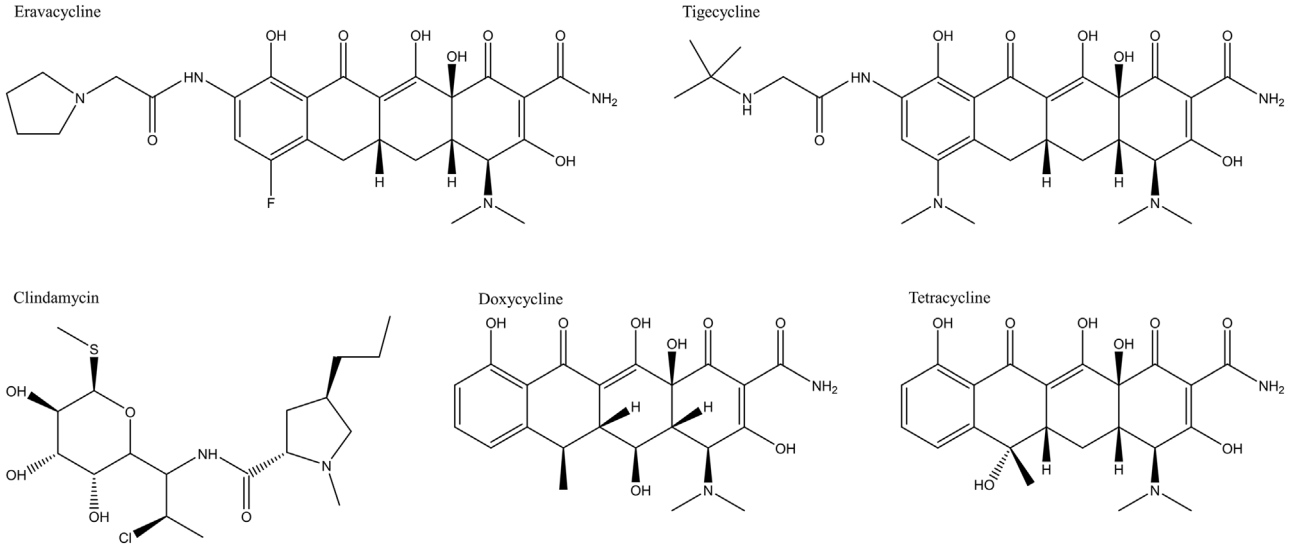

Fig. 1. Chemical structures of clindamycin, doxycycline, eravacycline, tetracycline, and tigecycline.

pronounced in clinical isolates and both compounds inhibited parasite propagation already during the first cycle. One possible explanation might be that parasites isolated from participants in endemic areas are already at the late ring stage and therefore the 3-day assay already continued into the second parasite replication cycle. However, this does not explain the variation observed between the different antibiotics tested. Difference in drug activities between laboratory strains and clinical isolates may be caused by mutations associated with culture adaptation or with adaptation of clinical isolates to constant immune and drug pressure in their environment. Long-term cultured *Plasmodium* parasites are known to alter cellular mechanisms to allow them to adapt more effectively to the in vitro environment, which may alter the drug's inhibitory activity [31]. These results emphasize the necessity to not only rely on results from laboratory strains, but additionally to test clinical isolates when developing novel antimalarials. Previous studies on clindamycin in laboratory strains and clinical isolates confirm the presented data here [32,33], but to our knowledge, no data on early activity of clindamycin against clinical isolates existed before this work. Nonetheless, this observation deserves further investigation.

Delayed parasite death, following eravacycline treatment and re-established viability after IPP supplementation, strongly indicates the apicoplast to be the target organelle, resulting in inhibition of isoprenoid precursor biosynthesis. Before confirmation of the apicoplast as the main target for tetracycline and lincosamide antibiotics in *P. falciparum*, the mitochondrion was proposed as the target for tetracyclines [34]. The comparatively high activity of eravacycline in the first cycle/3-day assay might be attributed to an additional effect on this organelle, but we did not investigate this further [7,24].

Currently, ACTs are the first-line treatment for uncomplicated *P. falciparum* malaria and patients with severe malaria are treated with intravenous artemisinin derivatives such as artesunate, according to WHO recommendations [35]. Symptoms caused by severe malaria are mimicked by a variety of other bacterial, parasitic, and viral diseases, which makes adequate diagnosis of severe malaria difficult in resource-limited health care systems. Combination therapies containing an antibiotic could therefore be used not only to treat malaria, but also bacterial co-infections [36]. For example, clindamycin – although slow acting – is an efficacious antimalarial treatment when given in combination with quinine as an ad hoc combination [37], the only treatment recommended in early pregnancy [9,38]. Tigecycline, a semisynthetic derivative of minocycline, was previously tested on clinical isolates of *P. falciparum* in Bangladesh and Gabon with an IC_{50} in the submicromolar range [19,39]. For eravacycline and tigecycline, only formulations for intravenous administration exist and would therefore limit their use in the treatment of severe malaria. An attempt was made to develop an oral formulation of eravacycline that was produced and investigated in a clinical study, but development was cut short because of poor outcome from low bioavailability [40].

In conclusion, tetracyclines have been in use for many decades as malaria interventions with high efficacy against *P. falciparum*. Eravacycline shows superior activity, against asexual blood stage *P. falciparum* parasites, when compared to currently used tetracyclines. These results show the potential of novel antibiotics as candidate drugs for antimalarial therapy.

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Author contributions

Study concept and design: EK, JH. Acquisition of data: EK, JH. Analyses and interpretation of data: EK, JH, AK. Drafting the manuscript: EK, JH, AK and all other authors reviewed the manuscript. Technical or material support: EK, JH, AK, BRA, RZM, MM, GM, AAA, STA, BM. Study supervision: JH, AK, MM, GM, AAA, STA, BM. All authors read and approved the final manuscript.

Competing interests

None declared.

Ethical approval

The study was approved by the Institutional Ethics Committee (CEI) of the Centre de Recherches Médicales de Lambaréné (CERMEL) under the number CEI-CERMEL015/2015.

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2.2. Chapter 2: Histone deacetylase inhibitors against *Plasmodium falciparum*.

Histone deacetylase inhibitors with high *in vitro* activities against Plasmodium falciparum isolates collected from Gabonese children and adults.

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Histone deacetylase inhibitors with high *in vitro* activities against *Plasmodium falciparum* isolates collected from Gabonese children and adults

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Histone deacetylase (HDAC) enzymes are targets for the development of antimalarial drugs with a different mode of action to established antimalarials. Broad-spectrum HDAC-inhibitors show high potency against *Plasmodium falciparum*, but displayed some toxicity towards human cells. Inhibitors of human HDAC6 are new drug candidates with supposed reduced toxicity to human cells and favorable activities against laboratory *P. falciparum* strains. We investigated the potency of 12 peptoid-based HDAC-inhibitors against asexual stages of *P. falciparum* clinical isolates. Parasites representing different genetic backgrounds were isolated from adults and children with uncomplicated malaria in Gabon. Clinical studies on (non-HDAC-inhibitors) antimalarials, moreover, found lower drug efficacy in children, mainly attributed to acquired immunity with age in endemic areas. Therefore, we compared the *in vitro* sensitivity profiles of adult- and child-derived isolates to antimalarials (HDAC and standard drugs). All HDAC-inhibitors showed 50% inhibitory concentrations at nanomolar ranges with higher activities than the FDA approved reference HDAC-inhibitor SAHA. We propose peptoid-based HDAC6-inhibitors to be lead structures for further development as antimalarial chemotherapeutics. Our results further suggest no differences in activity of the tested antimalarials between *P. falciparum* parasites isolated from children and adults.

Malaria is caused by protozoan parasites of the genus *Plasmodium* and is the most important parasitic disease worldwide. *Plasmodium falciparum* - the most virulent species - has become resistant to nearly all of the antimalarial compounds that are in clinical use[1–4]. In 2008, first evidence of artemisinin-resistant parasites was reported in western Cambodia[1,2]. There is a growing fear that resistance to artemisinin will continue to spread, especially to Sub-Saharan Africa. To keep up with resistance development of *P. falciparum*, new treatment options are constantly needed and chemical scaffolds with a new mode of action are of particular interest as they are less prone to be affected by cross-resistances. Histone deacetylase inhibitors (HDACi) are new antimalarial lead compounds known to inhibit multiple life cycle stages of *P. falciparum*[5,6].

In eukaryotes, deoxyribonucleic acid (DNA) is wrapped into tightly packed chromatin with the nucleosomes acting as the fundamental unit. Each nucleosome is composed of an octamer of two copies of four core histones. HDACs play an important role in the wrapping and unwrapping of DNA by increasing the affinity of histone octamers for DNA by removing acetyl groups from the side chain of specific lysine residues[7]. Inhibition of HDACs interferes with modulation of transcription, replication and DNA repair, and also the function of non-histone proteins[8]. Research on HDACi to target human diseases such as cancer has been ongoing for several decades and first compounds have entered the clinic as cancer therapies, proving the safety of this compound class for human

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use[7,9–12]. Recent data suggest HDACi as attractive drug candidates against parasitic diseases caused by *Trypanosoma, Toxoplasma, Schistosoma, Leishmania, and Plasmodium*[13–17]. The natural product apicidin was the first HDACi to be tested against *P. falciparum* and exhibited broad-spectrum antiprotozoal activity *in vitro* and *in vivo* in mice[18]. SAHA (suberoylanilide hydroxamic acid, vorinostat), romidepsin, belinostat, and panobinostat are all clinically approved HDACi used for cancer treatment and affect growth of various *Plasmodium* species including drug resistant *P. falciparum* strains[15]. Notably, HDACi were shown to be active against multiple life-cycle stages of *P. falciparum* including liver stages and gametocytes[12,19–21]. HDACi are promising lead structures for antimalarial drug development, but their use might otherwise be limited due to concomitant toxicity to human cells. This problem could be mitigated by developing inhibitors with relative or complete specificity towards plasmodial HDACs. In *P. falciparum*, up to six HDACs have been identified that share only a certain degree of sequence identity with human HDACs[22]. Little knowledge about structure and function of these enzymes in *P. falciparum* limits structure-based design of new inhibitors[23]. An alternative approach is to expand on human HDACi molecules, which are known to be less harmful to mammalian cells and drive their development towards parasite selectivity as well as anti-plasmodial activity. Selective inhibitors of human HDAC6 (hHDAC6), a class II enzyme, exert lower levels of cytotoxicity to human cells compared to HDAC class I inhibitors[24]. hHDAC6 targets in particular non-histone proteins (alpha-tubulin, Hsp90) and class II homologues that are also present in *P. falciparum* (PfHDAC2 and 3)[25–27].

Based on this assumption, a series of peptoid-based HDACi were developed[5,6]. These compounds are classical HDAC inhibitors that have a cap-linker-zinc binding group structure with a peptoid-based cap group (*N*-alkyl glycine derivatives). Preclinical screens of these candidates identified potent activity against blood stages of *P. falciparum* laboratory strains 3D7 and Dd2 and against *P. berghei* liver stages with promising parasite selectivity indices[5,6].

In vitro activity assessment of candidates against clinical *P. falciparum* isolates in early drug development can inform about the drug´s potency against parasite strains circulating in the target population in malaria endemic areas. *Plasmodium* parasites sampled from malaria patients are genetically very different from laboratory strains of *P. falciparum* that have been in *in vitro* culture for decades[28]. Additionally, the natural *P. falciparum* population is constantly exposed to host factors including antimalarial drug pressure and is therefore genetically highly diverse, and parasites may be intrinsically heterogenous in their susceptibility towards the molecule[29,30]. An additional layer of complexity results from clinical trials reporting different drug efficacies (of non-HDACi) against *P. falciparum* infections in adults and children[31–33]. These differences are mostly attributed to the partial immunity that is developed by the populations living in malaria endemic regions after multiple *P. falciparum* infections[34,35]. However, it has not been investigated if the parasites themselves isolated from children or adults show different drug susceptibility profiles in *in vitro* assays. Age-dependent immune responses that cause a difference in the number of *P. falciparum* strains co-infecting a single individual, also known as multiplicity of infection, could be one factor that provokes different susceptibility profiles *in vitro*.

Amongst the panel of previously published peptoid HDAC inhibitors, we selected 12 candidates with an IC50 below 100 nM against the laboratory strain 3D7 and different toxicity profiles for *in vitro* potency testing against *P. falciparum* isolates collected from infected individuals in Gabon, a country highly endemic for malaria[5,6,36–38]. We furthermore investigated the susceptibility of *P. falciparum* parasites isolated from children and adults towards standard antimalarial compounds and compared their activity profile.

Results

In total, 85 clinical isolates were collected from 52 children and 33 adults with uncomplicated *P. falciparum* malaria in Gabon. Clinical isolates were tested for their susceptibility to 12 HDACi candidates, 1 approved HDACi cancer drug as comparator and 8 known antimalarial compounds. Of the 85 assays, 53 (33 from children, 20 from adults) tests fulfilled strict quality criteria for successful growth and were included into further analysis of the inhibitor concentrations. The median age (IQR) of children and adults included was 3 years (2–4 years) and 21 years (19–50 years), respectively. The median parasitemia (IQR) in children and adults was 25,000 parasites/µl (9,120–62,192 p/µl) and 3,933 parasites/µl (1,802–14,193 p/µl), respectively.

In vitro activity of peptoid-based HDAC inhibitors against laboratory and clinical _P. falciparum_ isolates. We assessed *in vitro* activity of 12 peptoid-based HDACi candidates against *P. falciparum* isolates obtained from children and adults. The panel includes molecules from two generations of synthesis, no. 1 series (1a, 1d, 1g, 1h, 1i, 1u, and 1v) and no. 2 series (2c, 2g, 2h, 2i and 2m) (see Supplementary Fig. 1)[5,6]. Compounds were also tested against 3D7 laboratory strains to confirm activity of new compound production lots (Table 1). Compound 1 u was the most active HDACi candidate with a molecular activity of approx. 13 nM against *P. falciparum* strains isolated from both, children and adults (see Table 1). Compounds 1a, 1d, 1h, 1v, 2g, 2h, and 2i had good antiplasmodial activities with IC50 values in the double digit nanomolar range in children and adults. The IC50 values of drug candidates showed a wide range of activity (Supplementary Fig. 2). Some HDACi (1a, 1g, 1h, 1v, 2c, 2g, and 2h) were at least 2-fold more active against the parasite strains obtained from children compared to adults' strains, but differences did not reach the level of statistical significance.

Comparing drug susceptibility of _P. falciparum_ strains obtained from children and adults. To investigate if parasites obtained from semi-immune adults are less fit and more susceptible to drug testing *in vitro*, assays were done with standard and well-characterized antimalarial compounds tested against *P. falciparum* isolates from children and adults. All compounds confirmed potency against Gabonese parasite strains including chloroquine (Table 2). No significant differences in median IC50s between isolates from children and adults were observed for any of the drugs.

Quality control. Stability of dissolved test compounds during the study period of approximately 8 months was controlled by comparing IC50s against 3D7 assessed at project start and end. A fold increase of 1 was used to

Compound ID	Median IC50 (IQR) in nM						SI
	Clinical isolates – children & adults combined[a]	Clinical isolates – children only[a]	Clinical isolates – adults only[a]	3D7[b]	3D7[c]	Dd2[c]	
1a	47.1 (15.9–386.9)	42.6 (20.2–175)	116 (11.7–910)	21.3	20 ± 10	12 ± 8	>1295
1d	21.9 (8.8–97.8)	21.6 (9.1–71.4)	25.9 (7.1–127)	8.7	11 ± 4	14 ± 6	>850
1g	167 (34.6–1022)	117 (34.1–1268)	326 (37.7–1020)	49.4	25 ± 18	31 ± 12	>1613
1h	38.4 (11.0–96.3)	26.3 (12.6–92.8)	73.7 (10.0–97.3)	23.3	9 ± 3	15 ± 6	>520
1i	174 (61.7–406)	126 (71.0–413)	215 (45.9–405)	54.9	21 ± 16	33 ± 15	>642
1u	13.6 (4.4–89.8)	14.7 (4.6–76.9)	13.1 (3.1–146)	12.1	4 ± 1	1 ± 1	>2496
1v	65.1 (28.7–220)	54.3 (26.6–95.9)	110 (38.9–316)	9.8	14 ± 4	14 ± 4	>499
2c	410 (152–966)	287 (168–861)	619 (130–1116)	150.4	95 ± 15	ND	>526
2g	47.8 (9.6–215)	31.2 (9.6–231)	113 (6.8–200)	40.7	8.8 ± 3.2	ND	1483
2h	26.9 (5.2–94.5)	17.8 (5.6–93.3)	35.6 (3.8–95.5)	11.8	5.2 ± 3.6	ND	889
2i	32.1 (17.7–59.1)	28.0 (17.5–44.0)	40.8 (21.9–71.0)	11.3	9.7 ± 2.9	ND	64
2m	348 (191–760)	351 (193–834)	337 (178–535)	209.5	87 ± 44	ND	234
SAHA	267 (172–392)	276 (166–395)	267 (187–372)	187.3	139 ± 73	146 ± 22	>15

Table 1. Median IC50 and interquartile range (IQR) of HDACi candidates against *P. falciparum* clinical isolates, and laboratory strains 3D7 and Dd2. [a]Median (IQR). [b]Single measurement in duplicate at study start. [c]Previously published data (mean IC50)[5,6]. [d]SI Selectivity index: IC50 HepG2/IC50 3D7. ND: not determined.

Compound ID	Median IC50 (IQR) in nM			
	Clinical isolates – children & adults combined[a]	Clinical isolates – children only[a]	Clinical isolates – adults only[a]	P-value*
Chloroquine	15.4 (6.1–41.2)	12.3 (5.3–41.2)	15.7 (6.5–34.2)	0.853
Lumefantrine	2.8 (1.5–7.1)	3.3 (1.6–6.6)	2.4 (1.4–11.5)	0.662
Amodiaquine	2.2 (1.4–3.3)	2.8 (1.8–3.8)	1.7 (1.1–2.7)	0.009
Piperaquine	4.1 (2.8–7.2)	4.1 (2.8–7.5)	3.6 (2.8–5.8)	0.374
Pyronaridine	1.1 (0.4–2.1)	1.2 (0.4–2.3)	0.6 (0.4–1.7)	0.365
Ferroquine	1.8 (1.0–3.0)	1.9 (1.0–3.4)	1.4 (0.9–2.5)	0.159
Mefloquine	3.4 (2.2–5.1)	3.7 (2.2–5.5)	3.0 (2.3–5.1)	0.873
Atovaquone	0.3 (0.2–0.5)	0.4 (0.2–0.5)	0.3 (0.2–0.5)	0.686

Table 2. Median IC50 and interquartile range (IQR) of standard antimalarial compounds against *P. falciparum* clinical isolates obtained from children and adults. [a]Median (IQR). *Differences in activities of clinical isolates of adults and children were compared by Mann-Whitney U test.

determine the stability of a compound at study end. Drug instability was not observed in any of the standard antimalarials or SAHA. Except for 1h, 2g and 2m, all HDACi candidates were at least 1.6-fold less active at the study end when controlled with 3D7 (Supplementary Fig. 3). Strains from children and adults were equally sampled over time (data not shown).

Discussion

The development of new drugs and particularly of those with novel targets and modes of action is urgently needed to compete with the development of resistance by *P. falciparum* to current antimalarials. So far, mainly broad-spectrum HDACi have been investigated as potential antiplasmodial drugs, since several human HDAC

homologues have been characterized in *P. falciparum*. Anti-cancer HDACi, already FDA approved, have been tested against *P. falciparum* and have been shown to effectively kill the parasites *in vitro* and also showed high activity against *P. berghei* in *in vivo* mouse models at sub-micromolar concentrations, but are known to cause a certain level of toxicity to human cells[6,18,20]. HDACi affect multiple eukaryotic cell functions, including non-histone related pathways where class IIb HDACs (e.g. hHDAC6) are involved[24]. Homologues of hHDAC6 proteins have been identified in *P. falciparum* and targeting class II HDACs might be a possibility to circumvent toxicity in humans[24].

We tested the 12 most promising peptoid-based HDACi candidates against clinical *P. falciparum* isolates from Gabon and confirmed previous results in laboratory strains which showed the HDAC6 inhibitor 1u, *N*-(2-(Cyclohexylamino)-2-oxoethyl)-*N*-(4-(hydroxycarbamoyl)benzyl)-4-isopropylbenzamide, to be the most potent HDACi with a mean IC50 of 4 nM and high selectivity towards the parasites[5]. Here, our results also showed compound 1u to be the most active HDACi against clinical *P. falciparum* isolates. The high drug activity of 1u may have been obtained by replacement of the *N*, *N*-dimethylamino group, from molecule 1h, with a less polar isopropyl group[5]. The results of our investigation in clinical isolates resemble those previously obtained in laboratory strains[6,12], confirming that these compounds are equally active against a parasite population of high genetic diversity under selection pressure by the currently used antimalarial drugs[30,37]. All compounds were more active than the comparator broad-spectrum HDACi SAHA (vorinostat), which we found to have an IC50 similar to that reported in the literature[15,39]. HDACi candidates, including potent compound 1u, seem to suffer from chemical instability in solution that is less pronounced for SAHA. Loss in activity over time may also explain the observed wide range of IC50 data, that otherwise could be interpreted as suggesting towards intrinsic parasite resistance.

Differences in *in vivo* drug activities against *P. falciparum* infections between children and adults are usually attributed to the well-known acquisition of immunity after repeated infections in high endemic regions[32–34]. There is evidence that multiplicity and diversity of strains differs between adults and children[40–42]. However, to the best of our knowledge, there has been no study that specifically looked at differences in antiplasmodial *in vitro* drug activity between children and adults. Overall, our results suggest no significant differences in *in vitro* activity of HDACi and standard antimalarials in *P. falciparum* strains collected from children and adults. We found only amodiaquine to potentially show such a difference, a drug to which the parasite population is constantly exposed, although this finding did not hold under correction for multiple testing. Whether this difference is a true finding has to be further examined in future investigations. Overall, however, our results suggest no significant differences in the *in vitro* activity of either HDACi or standard antimalarials against *P. falciparum* strains collected from adults and children.

The standard antimalarials we tested were all highly active against clinical *P. falciparum* isolates from Gabon with no observed resistance except in chloroquine, to which 13 outliers showed an IC50 greater than 40 nM. First line treatment in Gabon changed from chloroquine to the artemisinin combination therapies (ACTs) artesunate-amodiaquine or artemether-lumefantrine in 2003[43]. Despite this change, chloroquine resistance appears to remain high, but a tendency towards declining chloroquine resistance can be extrapolated from the *in vitro* data[43–46]. This might indicate that the full reversal of chloroquine sensitivity can be observed in the future, as has occurred in other malaria endemic regions[47]. Only a few outliers were present for the other standard antimalarials, which may not necessarily be attributed to resistance. Amodiaquine and lumefantrine are first-line partner drugs to artemisinin-derivatives in Gabon, but fortunately signs of resistance cannot be detected[48].

Our analysis of clinical *P. falciparum* isolates from Gabon confirm the results from previous work showing HDACi candidate 1u to be highly active and indeed more potent than the comparator cancer drug SAHA. These inhibitors targeting class II HDAC proteins are potential lead structures for further development as antimalarial chemotherapeutics with promising selectivity towards *Plasmodium* parasites, but require improvement of chemical stability. Differences in the immune status to malaria between adults and children seemed not to affect the observed drug potency against *P. falciparum*.

Methods

Clinical isolate sampling. In total, 85 participants with uncomplicated malaria were enrolled between October 2017 and June 2018 residing in Lambaréné and surrounding villages in Gabon. Informed consent was obtained from study participants or the legal representative, if minor. The study was approved by the Institutional Ethics Committee of the Centre de Recherches Médicales de Lambaréné (CERMEL) with the number CEI-CERMEL015/2015. Inclusion criteria were written informed consent, age either 1 to 5 years (children cohort) or 18 years and older (adult cohort), and *P. falciparum* monoinfection with a parasitemia above 1000 parasites/µl assessed by Giemsa-stained thick blood smear. To obtain clinical *P. falciparum* isolates, a venous blood sample was taken in a lithium heparin tube/EDTA tube and processed in the *in vitro* drug sensitivity assay within approximately 6 hours. All methods were performed in accordance with relevant guidelines and regulations.

Parasite culture. *Plasmodium falciparum* laboratory strain 3D7 (chloroquine-sensitive) was maintained in continuous *in vitro* culture as previously described[49]. Parasites were kept in complete culture medium (RPMI 1640, 25 mM 4-(2-hydroxyethyl) piperazine-N′-(4-butanesulfonic acid), 2 mM L-glutamine, 50 µg/mL gentamicin, and 0.5% w/v albumax) at 37 °C, at 2.5% hematocrit in a candle jar with daily change of medium. Synchronization was performed by 5% sorbitol twice a week[50].

Compounds. All compounds were dissolved in sterile DMSO if not otherwise stated. In total,12 candidate HDAC inhibitors were tested and 1g, 1h, 1i, 1u, 2c, 2g, 2i, and 2m were dissolved to reach a stock concentration of 25 mM and 1a, 1d, 1v, and 2h were prepared at 100 mM (chemical structures see Supplementary Fig. 1). SAHA (Hycultect), an approved HDAC inhibitor served as a control and was dissolved at 100 mM in DMSO. All

comparator antimalarial compounds were obtained from Sigma-Aldrich if not otherwise stated. Lumefantrine, mefloquine hydrochloride, ferroquine (Sanofi-Synthelabo), were prepared at 12.5 mM stock concentration; 100 mM stocks were made for amodiaquine dihydrochloride dihydrate, pyronaridine tetraphosphate, piperaquine tetraphosphate tetrahydrate was dissolved at 6.25 mM; and atovaquone (GlaxoSmithKline) at 25 mM. Chloroquine diphosphate salt was dissolved in double-distilled water at 100 mM. All stocks were freshly prepared for the study and stored at −20 °C. Maximum concentration of solvent DMSO in the *in vitro* assay was 0.01% and did not interfere with parasite growth in pilot experiments.

Drug sensitivity assay. Drug sensitivity assays were performed according to standard procedures[46]. Briefly, 96 well-plates were pre-dosed with a threefold serial dilution of the respective drug in complete culture medium to obtain the following range of concentrations: chloroquine, 1.2 to 1000 nM; lumefantrine, 0.4 to 1000 nM; amodiaquine, 0.4 to 100 nM; piperaquine, 0.3 to 250 nM; pyronaridine, 0.02 to 50 nM; ferroquine, 0.08 to 200 nM; mefloquine, 0.7 to 500 nM; atovaquone, 0.02 to 20 nM; 1a, 4.1 to 10000 nM, 1d, 1.2 to 3000 nM; 1g, 12.3 to 10000 nM; 1h, 0.34 to 750 nM; 1i, 12.3 to 10000 nM; 1u, 0.34 to 750 nM; 1v, 2.7 to 2000 nM; 2c, 12.3 to 15000 nM; 2g, 0.34 to 750 nM; 2h, 1.2 to 1000 nM; 2i, 0.41 to 300 nM; 2m, 6.9 to 5000 nM; SAHA, 12.3 to 15000 nM. Ring-stage parasites from the laboratory strain 3D7 and clinical *P. falciparum* isolates were adjusted to a parasitemia of 0.05% with 0^+ erythrocytes and the hematocrit was set to 1.5% in a total volume of 225 µl per well. After 72 hours of incubation at 37 °C, plates were freeze-thawed three times and analyzed by measurement of *P. falciparum* histidine-rich protein 2 (HRP2) with an enzyme-linked immunosorbent assay (ELISA)[51]. Only assays with successfully grown and propagating parasites reflected by a 1.5 OD increase between full and no inhibition within the 72 hours were included in the IC50 analysis. All experiments were done in duplicates. To control quality and stability, all compounds were additionally tested against the 3D7 laboratory strain once before and three times after testing of clinical isolates.

Statistics. Individual inhibitory concentrations were determined by non-linear regression analysis of log-concentration-response curves using the drc-package v3.0-1 of R version 3.4.2. Data for the clinical isolates was presented using the median 50% inhibitory concentration (IC50) and the interquartile range (IQR). Correlations between IC50 values of clinical isolates of children and adults of the different standard antimalarials and HDACi were calculated using the Mann-Whitney U (nonparametric) test in JMP v14.0.0 software. IC50 values, calculated by R, to be over the highest tested drug concentration were substituted with the highest tested concentration value if parasites were dead at the highest tested drug concentration (indicated by a low OD value of confirmed dead parasites from the same patient).

Ethics approval and consent to participate. The study was approved by the Institutional Ethics Committee (CEI) of CERMEL with the number CEI-CERMEL015/2015.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Study concept and design: E.K., J.H. Acquisition of data: E.K., J.H. Analyses and interpretation of data: E.K., J.H., A.K. Drafting the manuscript: E.K., J.H., A.K. and all other authors reviewed the manuscript. Technical or material support: E.K., J.H., R.Z.M., A.K., M.M., G.M., M.K.W.M., F.K.H. Study supervision: J.H., A.K., M.M., G.M. All authors read and approved the final manuscript.

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The authors declare no competing interests.

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2.3. Chapter 3: Antiplasmodials against *Schistosoma mansoni*.

Evidence for *in vitro* and *in vivo* activity of the antimalarial pyronaridine against Schistosoma.

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RESEARCH ARTICLE

Evidence for *in vitro* and *in vivo* activity of the antimalarial pyronaridine against *Schistosoma*

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Abstract

Background

Schistosomiasis is highly prevalent in Africa. Praziquantel is effective against adult schistosomes but leaves prepatent stages unaffected—which is a limit to patient management and elimination. Given the large-scale use of praziquantel, development of drug resistance by Schistosoma is feared. Antimalarials are promising drugs for alternative treatment strategies of Schistosoma infections. Development of drugs with activity against both malaria and schistosomiasis is particularly appealing as schistosome infections often occur concomitantly with malaria parasites in sub-Saharan Africa. Therefore, antiplasmodial compounds were progressively tested against Schistosoma *in vitro*, in mice, and in a clinical study.

Results

Amongst 16 drugs and 1 control tested, pyronaridine, methylene blue and 5 other antimalarials were highly active *in vitro* against larval stage schistosomula with a 50% inhibitory concentration below 10 μM. Both drugs were lethal to *ex vivo* adult worms tested at 30 μM with methylene blue also active at 10 μM. Pyronaridine treatment of mice infected with *S. mansoni* at the prepatent stage reduced worm burden by 82% and cured 7 out of 12 animals, however in mice adult stages remained viable. In contrast, methylene blue inhibited adult worms by 60% but cure was not achieved. In an observational pilot trial in Gabon in children, the antimalarial drug combination pyronaridine-artesunate (Pyramax) reduced *S. haematobium* egg excretion from 10/10 ml urine to 0/10 ml urine, and 3 out of 4 children were cured.

Conclusion

Pyronaridine and methylene blue warrant further investigation as candidates for schistosomiasis treatment. Both compounds are approved for human use and evidence for their potential as antischistosomal compounds can be obtained directly from clinical testing. Particularly, pyronaridine-artesunate, already available as an antimalarial drug, calls for further clinical evaluation.

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Trial registration

ClinicalTrials.gov Identifier NCT03201770.

Author summary

Praziquantel is still the only drug in use for the treatment of all *Schistosoma spp*. and is exclusively active against the adult life cycle stage, since schistosomes in the prepatent period of up to eight weeks are not affected by the drug. Although resistance to praziquantel has not been confirmed and its existence remains controversial, some countries have identified clinical schistosome isolates with reduced sensitivity to praziquantel, after deployment in mass drug administration programs. The need for a new antischistosomal compound is urgent, ideally exhibiting broad activity against all stages of the parasite's life cycle present in humans. After testing a series of antiplasmodial compounds, the authors found that several compounds also exhibited antischistosomal activity at various life cycle stages of the worms, including pyronaridine and methylene blue, both compounds already approved for human use. A pilot trial with pyronaridine-artesunate done in Gabon showed the first promising results against Schistosoma infections.

Introduction

Schistosomiasis is an infectious disease caused by parasitic flatworms of the genus *Schistosoma*. WHO estimated that approximately 100 million people were treated for schistosomiasis in 2018, with about 90% of cases occurring in Africa, and approximately 290 million people receiving preventive treatment [1]. Praziquantel (PZQ), the only drug for the treatment of all *Schistosoma spp*., including *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*, is exclusively active against the adult life cycle stage. Schistosomes in the prepatent period of up to eight weeks are not affected which is a limit to schistosomiasis control and elimination efforts by PZQ mass administration [2,3]. Resistance to PZQ has not been confirmed and its existence remains controversial, yet, clinical schistosome isolates with reduced sensitivity have been identified in Egypt and Senegal after PZQ deployment in mass drug administration programs [4–6]. The need for a new antischistosomal compound is urgent, optimally exhibiting broad activity against all stages of the parasite's life cycle present in humans.

Drug repurposing is a rapid and efficient strategy to identify compounds with new therapeutic targets [7]. Interestingly, schistosomes and malaria parasites both degrade blood/hemoglobin and depend on intracellular mechanisms to protect themselves from heme toxicity; a molecular pathway inhibited by several antimalarial compounds [8,9]. In Africa, individuals are often co-infected with *Schistosoma spp*. and *Plasmodium spp*. since both parasites have a large geographical overlap in endemicity. Antiplasmodial compounds approved for use in humans may serve as "low hanging fruits" for the development of new antischistosomal interventions either as antimalarial treatments with an add-on effect against schistosomes or as a starting point for a schistosomiasis drug development program.

Indeed, antimalarials such as artesunate, artemether, and mefloquine have been tested, as a monotherapy or in combination, in clinical trials for their antischistosomal potency [10–12]. Of particular interest is their activity against young schistosomes, as identified in preclinical studies [13,14]. Differences in the design and quality of the studies carried out make it difficult to draw an overall conclusion on the antischistosomal efficacy of artemisinins and mefloquine

[15,16]. However, given together with PZQ, all life cycle stages in humans could be targeted [17,18]. Artemisinin and related derivates (i.e. artesunate, artemether) constitute the standard of care for malaria treatment that is an artemisinin-based combination therapy (ACT). An ACT is formed by an artemisinin derivate combined with a long-acting partner drug of a different drug class. Amongst the six ACTs on the market, pyronaridine-artesunate was registered recently with a granule's formula also available for treatment of small children. Indication towards an add-on antischistosomal effect of an ACT drug component could be an interesting starting point for further clinical evaluation. Several antimalarials have also been tested preclinically for activity against *Schistosoma* within individual small scale studies and/or library screenings projects [19,20]. Here, we tested a series of antiplasmodial compounds head-to-head in a hierarchical workflow for their antischistosomal activity and chose the most promising compounds for *in vivo* testing [21].

Results

In total, 16 compounds with reported antiplasmodial activity were tested for activity against *S. mansoni*. The drugs were tested in a hierarchical workflow to downselect candidates with antischistosomal activity starting with *in vitro* drug sensitivity assays on human larval stage schistosomes, schistosomula, (step 1) followed by testing of *ex vivo* adult worms (step 2), and promising compounds were assayed in *S. mansoni* infected mice (step 3). An important aspect was to identify molecules with activity against juvenile worms. This stepwise approach helps to reduce animal testing. In addition, preliminary data of an observational study assessing the activity of pyronaridine-artesunate against urinary schistosomiasis in children in Gabon were analyzed (step 4).

In vitro schistosomula assays (step 1)

Most of the compounds were registered antimalarial drugs of diverse pharmacological classes including antibiotics (S1 and S2 Tables) and were tested head-to-head within the same assay set-up. In the analysis only experiments passing the following quality criteria were included: schistosomula viability $>$ 80% after 7 days and the 50% inhibitory concentration (IC50) of mefloquine below 2 μM (S1 Fig). Of the 16 compounds tested, 7 had an IC50 below 10 μM (Fig 1). None of the antibiotics were active (Table 1). The positive control drug mefloquine was confirmed to be highly active and, interestingly, together with methylene blue, were the only 2 drugs with a rapid onset of activity (IC50 below 10 μM after 72 h).

There is no *gold standard* methodology to determine the worms´ viability after drug exposure to report a compound´s antischistosomal activity. Microscopy based classification is dependent on the reader´s subjective judgement. To minimize observer bias, every assay was not only analyzed by microscopy, but after 7 days drug exposure outcomes were concomitantly analyzed by resazurin and lactate assays. Resazurin measurement [22] reflects aerobic respiration and lactate detection is a surrogate marker for glucose metabolism, [23] both indicating cell viability. Overall, IC50s obtained by microscopy and by resazurin assay on day 7 were in agreement for active compounds with lower IC50s (Table 1 and S2 Fig). Pyronaridine was active, but only one experiment resulted in a sigmoidal dose-response curve allowing for the calculation of the IC50 at the concentration range tested (S5 Fig). Interestingly, the lactate assay identified compounds with a rather rapid onset of inhibition(IC50 $\leq$ 10 μM after 72 h drug exposure) as found by microscopy at 72 h (mefloquine, methylene blue, primaquine (IC50 of 12.7 μM). Drugs which exerted an IC50 below 10 μM after 7 days only, (amodiaquine, chloroquine) were not found active by lactate assay.

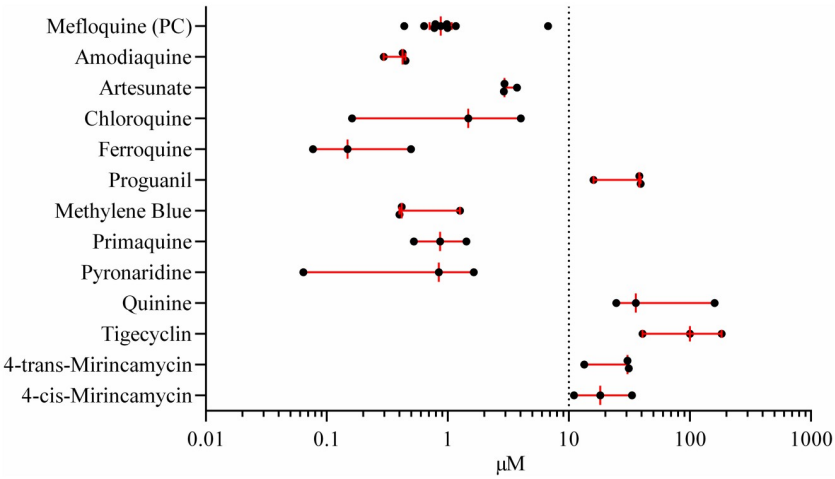

Fig 1. *In vitro* schistosomula assays (step 1). Dot plot of individual IC50 values of 12 drugs and mefloquine control is shown. Each compound was tested in 3 independent, *in vitro* schistosomula experiments (except for MQ, N = 9) and individual IC50s (filled circles) are shown. Viability of schistosomula after 7 days drug exposure was assessed by microscopy. For four drugs (atovaquone, cycloguanil, doxycycline, clindamycin) the IC50 could not be calculated as no inhibition was observed. The IC50 is given in μM. Bar and lines indicate median IC50 and IQR. Dotted line: Threshold for drug activity is an IC50 < 10 μM. IQR: Interquartile range.

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Table 1. Assay-dependent IC50s of compounds against schistosomula (step 1).

Drug	Microscopy at 72 h	Microscopy day 7	Resazurin day 7	Lactate day 7
Mefloquine	4.9 (2.7–7.8)	0.8 (0.7–1.1)	0.7 (0.6–1.9)	1.7 (0.7–4.1)
Amodiaquine	no inhibition	0.4 (0.3–0.4)	0.2 (0.1–0.3)	no inhibition
Artesunate	28.4 (14.3–30.1)	2.9 (2.9–3.7)	1.7 (1.7–3.4)	12.5 (7.9–16.6)
Atovaquone	no inhibition	no inhibition	no inhibition	no inhibition
Chloroquine	no inhibition	1.5 (0.2–4.0)	2.7 (0.4–4.2)	no inhibition
Ferroquine	no inhibition	0.1 (0.1–0.5)	0.6 (0.5–0.8)	no inhibition
Cycloguanil	no inhibition	no inhibition	no inhibition	no inhibition
Proguanil	71.6 (41.3–99.6)	38.2 (16.0–39.3)	15.7 (19.0–13.6)	45.9 (33.3–58.4)
Methylene blue	1.4 (1.9–3.7)	0.4 (0.4–1.3)	#	0.3 (0.3–0.4)
Primaquine	12.7 (5.2–45.3)	0.9 (0.5–1.4)	0.5 (0.2–1.0)	6.1 (5.0–7.2)
Pyronaridine	71.6 (41.3–99.6)	0.8 (0.1–1.6)	0.5*	1.0*
Quinine	no inhibition	35.9 (24.7–160.2)	26.3 (21.0–70.0)	no inhibition
Doxycycline	no inhibition	no inhibition	no inhibition	no inhibition
Clindamycin	no inhibition	no inhibition	no inhibition	no inhibition
cis-Mirincamycin	33.2 (22.4–45.3)	18.2 (11.0–33.2)	13.4 (12.4–29.3)	18.7 (10.0–41.3)
trans-Mirincamycin	33.2 (31.2–67.2)	30.6 (13.4–31.2)	19.3 (17.9–28.5)	33.6 (17.3–35.7)
Tigecycline	125.0 (59.5–125.0)	100.0 (40.1–83.0)	no inhibition	no inhibition

In vitro viability of *S. mansoni* schistosomula was assessed by microscopy after 72 h and at day 7 after drug exposure, respectively, and by resazurin assay and lactate assay after 7 days only. Median (IQR) in μM is reported. Mefloquine served as positive control.

[#] could not be measured due to drug colour interference with the reader.

[*] IC50 value from one assay. Each drug was independently tested at minimum three times.

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Ex vivo adult worm assays (step 2)

Methylene blue and pyronaridine were further tested for their *in vitro* activity against adult *S. mansoni*. Methylene blue at 10 μM for 7 days was highly active, whereas pyronaridine was active at 30 μM (Table 2). Both drugs rapidly killed all worms within 24 h at 30 μM. Irreversibility of drug effects was controlled by continued culture for 7 days after drug/medium replacement by fresh medium only (S3 Fig and S1 Videos).

In vivo drug activity in *S. mansoni*-infected mice (step 3)

Pyronaridine and methylene blue were chosen for further evaluation based on their *in vitro* activities against schistosomula, adult worms, and review of the literature (S2 Table) *In vivo* potency of compounds was tested against mice infected with *S. mansoni* at the juvenile stage (14 days after mice were infected with *S. mansoni*) as well as against the adult stage (9 weeks after *S. mansoni* infection). Artesunate and praziquantel were included as positive control drugs with known activity against juvenile schistosomes and adult worms, respectively [24,25]. Pyronaridine (500 mg/kg/day given for 5 days) was highly active against juvenile stages with a cure rate of 58%, whereas methylene blue (100 mg/kg/day given for 5 days) did not show an effect. For pyronaridine, the number of worms was substantially reduced (82%) reaching a comparable efficacy level as artesunate (79%) (Fig 2A and Table 3). No schistosome eggs (liver granulation) were observed in the livers of pyronaridine treated mice in contrast to methylene blue treated mice which displayed eggs in numbers as high as in the control mice (S4 Fig). Although cure was not achieved, methylene blue reduced the adult worm burden considerably (57%), which was not observed for pyronaridine (Fig 2B and Tables 4 and S4).

Observational pilot study in Gabonese patients (step 4)

Ultimately, data from *S. haematobium* infected children who received pyronaridine-artesunate within a non-randomized, observational, study for uncomplicated malaria were analyzed for efficacy of pyronaridine-artesunate against urinary schistosomiasis. This preliminary proof-of-concept exploratory analysis was done for 6 children at the trial site CERMEL/Gabon. Egg excretion was assessed 28 days after treatment start in 4 children (2 children were lost to follow-up) (S3 Table). Median number of excreted eggs was 10/10 ml of urine (IQR: 5–1528) at the day of treatment start and 0/10 ml (IQR: 0–1570) 28 days later. Cure was achieved in 3 out of 4 children (Fig 3). Microhematuria and proteinuria were resolved in 50% and excretion of leucocytes was resolved in 75% of the children.

Discussion

Schistosomiasis treatment, prevention, and control suffers from a lack of approved treatment options besides praziquantel as the standard of care. As concomitant schistosome and malaria parasite infections are frequent in Africa, a comprehensive assessment of antimalarial drugs for their potency against schistosomiasis could be an efficient starting point for the development of new intervention strategies [26].

We tested a series of compounds with known antiplasmodial activity for their inhibitory effect against *S. mansoni*. Out of the 16 compounds investigated plus mefloquine and artesunate used here as controls, 9 drugs (including mefloquine, artesunate, amodiaquine, and atovaquone) have been previously screened for their activity against various life cycle forms of *S. mansoni*. To consolidate evidence of a molecule´s potential for further antischistosomal drug development, we did a head-to-head *in vitro* evaluation of antimalarials against larval, prepatent schistosomes, and a selection of candidates were further tested *in vitro* against *ex vivo*

Table 2. Drug activity against *ex vivo* adult worms (step 2).

Drug	Concentration in μM	No. of worms tested	% affected	% dead
No drug	NA	12	0	0
DMSO	0.1%	10	0	0
Praziquantel	1	12	0	100
	5	17	35	59
Methylene blue	10	12	0	100
	30	12	0	100
	30*	6	0	100
	5	15	0	60
Pyronaridine	10	11	45	55
	30	12	0	100
	30*	6	0	100

Methylene blue, pyronaridine, and praziquantel (positive control) were exposed to the respective concentrations for 7 days (* for 24 h) followed by a 7 days drug wash-out *in vitro* culture."No drug" and DMSO were the negative controls. Pooled data obtained from 3 experiments per drug are displayed.

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adult worms and *in vivo* in mice. The first stage-gate was set to an IC50 of 10 μM against schistosomula *in vitro* to [21] downselect the number of candidates and to reduce animal involvement. Our approach confirmed the reported antischistosomal activity of mefloquine [27], artesunate, and primaquine [28] when schistosomula stages were exposed for 7 days and found chloroquine and ferroquine highly active. We confirmed the insensitivity of schistosomes to antibiotics although some interesting antischistosomal effects were observed for both isomers of mirincamycin.

Amodiaquine and methylene blue, the latter so far only used for staining and visualization of the worms [29,30] were active against *in vitro* schistosomula. We also confirmed activity of pyronaridine against schistosomula and found both, pyronaridine and methylene blue, rapidly

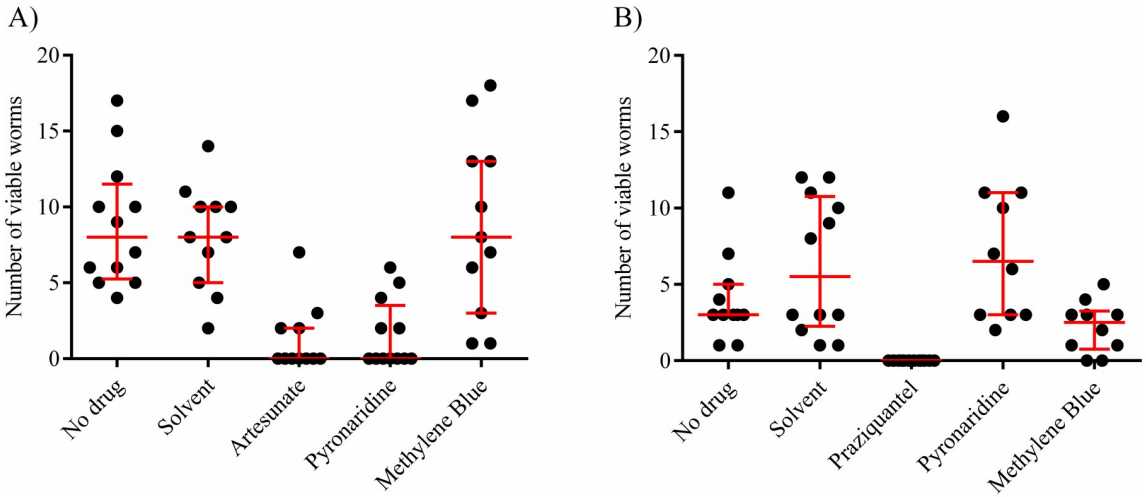

Fig 2. Drug activity in *S. mansoni*-infected mice (step 3). Mice were treated with respective compounds after A) 14 days (juvenile worms) and B) 9 weeks (adult worms) of *S. mansoni* infection, respectively. Mice were killed and adult worms were recovered and counted. MB: methylene blue, PY: pyronaridine, AS: artesunate, PZQ: praziquantel. Each data point (filled circle) represents one mouse. Thick bar: median, whiskers: IQR. A statistically significant difference ($P < 0.05$) to no drug control was detected for A) AS and PY, and for B) PZQ, respectively. Results from two independent experiments are shown for A and from one experiment for B.

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Table 3. Activity against juvenile *S. mansoni* in mice.

Juvenile worms (2 weeks old)								
Drug	Dose in mg/kg	No. of mice	No. of worms/mouse recovered Median (IQR)			Cure in %	WBR in %	LGS Median (IQR)
			Total	Males	Females			
No drug	NA	12	8.0	5.5	2.0	NA	0	2.5
			(5.3–11.5)	(4.0–9.0)	(1.0–4.5)			(2.0–3.0)
Solvent	NA	12	9.0	8.0	1.5	NA	0	3.0
			(5.5–10.8)	(4.0–9.8)	(0–2.8)			(2.3–3.0)
Artesunate	300	12	0.0	0.0	0.0	58	79	0.0
			(0.0–2.8)	(0.0–2.8)	(0–0.8)			(0.0–0.8)
Pyronaridine	500	12	0.0	0.0	0.0	58	82	0.0
			(0.0–3.5)	(0–1.8)	(0–1.0)			(0.0–0.8)
Methylene blue	50	12*	8.0	7.0	2.0	0	1	3.0
			(3.0–13.0)	(2.0–10.0)	(0.0–4.0)			(3.0–3.0)

Mice infected with *S. mansoni* for 2 weeks (worms are in the juvenile stage) were treated with methylene blue (MB) or pyronaridine (PY). Negative control treatment: No drug and solvent, positive control treatment: Artesunate (AS). WBR is referred to the "No drug" group. WBR: Worm burden reduction, LGS: Liver granulation score.

*1 mouse died during the treatment week. Compounds were tested in two independent experiments.

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inhibiting *ex vivo* adult worms tested *in vitro*. Despite similar assay conditions (24 h approx. 30 μM drug exposure), Panic et al. [19] found methylene blue and pyronaridine not efficacious against *ex vivo* adult worms although these results are not further detailed, but consequently refrained from mouse testing. This discrepancy in assay outcomes underlines the need of standardized and validated assay methodology and repetitive antischistosomal testing of molecules

Table 4. Activity against adult *S. mansoni* in mice.

Adult worms (9 weeks old)								
Drug	Dose in mg/kg	No. of mice	No. of worms/mouse recovered Median (IQR[a])			Cure in (%)	WBR in %	LGS Median (IQR[a])
			Total	Males	Females			
No drug	NA	6	6.0	4.0	2.0	NA	0	3.0
			(2.5–15.0)	(1.8–8.8)	(0.8–6.3)			(3.0–3.0)
Solvent	NA	6	10.5	5.5	5.0	NA	0	3.0
			(6.3–12.0)	(4.0–6.3)	(2.3–5.3)			(2.8–3.0)
Praziquantel	500	6	0.0	0.0	0.0	100	100	3.0
			(0.0)	(0.0)	(0.0)			(2.8–3.0)
Pyronaridine	500	6*	11.0	6.0	4.0	0	0	3.0
			(8.5–13.5)	(5.5–7.0)	(3.0–7.0)			(3.0–3.0)
Methylene blue	50	6*	3.5	2.5	1.0	0	57	3.0
			(3.0–4.8)	(2.0–4.5)	(0.3–1.0)			(2.3–3.0)

Mice infected with *S. mansoni* for 9 weeks (worms are in the adult stage) were treated with methylene blue (MB) or pyronaridine (PY). Negative control treatment: No drug and solvent, positive control treatment: Praziquantel (PZQ). WBR is referred to the "No drug" group. WBR: Worm burden reduction, LGS: Liver granulation score.

* 1 mouse died during the treatment week.

[a] Interquartile range: IQR. Results from one experiment are shown (a second experiment did not produce comparable infection rates of mice and results were excluded here but can be found in S4 Table).

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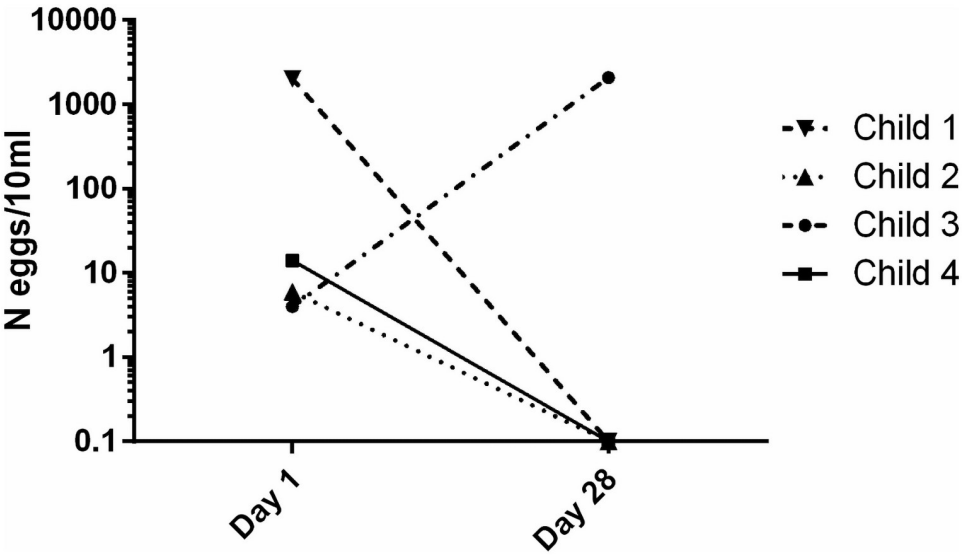

Fig 3. Observational study in Gabonese patients (step 4). Individual treatment response. Children excreting *S. haematobium* eggs were treated with pyronaridine-artesunate starting at day 1. After 28 days, urines were analyzed again and number of eggs/10 ml urine was reported.

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by various groups. This also includes harmonization of culture media as serum binding properties of compounds may impact drug activity [31].

Methylene blue, a registered drug to treat methemoglobinemia caused by drug or toxin exposure and in development as an antimalarial [32,33], and pyronaridine, approved as a malaria treatment in a fixed combination with artesunate (Pyramax) were further tested in mice infected with *S. mansoni* at the juvenile or adult stage, respectively, at the time of treatment administration. We found pyronaridine to be active against the juvenile forms with little to no activity against adult worms, which is in line with Keiser et al. [13]. No eggs were found in the livers of dissected mice treated with pyronaridine indicating a high potency against young life cycle forms. Methylene blue, on the other hand, was very potent against the adult worms *in vivo*. Methylene blue has been found to act by preventing the polymerization of heme into hemozoin in *Plasmodium* parasites [34] and studies on the mode of action of pyronaridine report inhibition of β-hematin production and glutathione-dependent heme degradation [35,36]. This makes both compounds interesting candidates for further development of novel schistosomiasis treatments since they have the potential to inhibit vital heme-related processes of *P. falciparum* and potentially also of *S. mansoni*.

Schistosomiasis drug discovery programs are still hampered by the lack of robust, scalable, and quantitative read-outs of schistosome viability after drug exposure that limits phenotypic whole organism screens. We recently identified the energy metabolism of schistosomes as a new target to quantify the efficacy of a test compound [23] which may prove to be especially helpful for schistosomula screens. Viable schistosomes rely on glycolysis of glucose to generate energy and excrete lactate that accumulates in the surrounding medium. Interestingly, drugs with a rapid onset of activity (exerting 72 h IC50 by microscopy below/around 10 μM) were also identified active by lactate assay and compounds with lower activities were confirmed by lactate assay outcomes (either high IC50 values or no IC50 could be calculated). Detection of lactate levels might reflect how rapidly the worm´s glycolysis is inhibited and could indicate early on set of drug activity. Resazurin assays report worm viability/death at the end of drug

exposure and the assay reliably identified active compounds when compared against microscopy after 7 days drug exposure. Another hurdle to drug development is the limited translation of efficacy data derived from animal models into clinical efficacy. PZQ, the only schistosomiasis drug, requires an oral dose of 400 mg/kg in *S. mansoni* infected mice [13], whereas only 40 mg/kg is administered to humans. Methylene blue and pyronaridine were tested active at 100 mg/kg and 500 mg/kg respectively, in *S. mansoni* mice. Although for malaria treatment both drugs are used at considerably lower doses (approximately 20 mg/kg for 3 days [37] for methylene blue and 1 tablet of 180 mg pyronaridine (plus 60 mg artesunate) once daily for 3 days for children with a weight of 20 to max. 24 kg), their effectiveness against schistosomes requires testing in clinical trials.

Pyronaridine is the long acting partner drug of artesunate in the artemisinin-based combination therapy (ACT) that was recently recommended by WHO for the treatment of uncomplicated malaria [38]. Pyronaridine has an estimated terminal elimination half-life of around 14 days [39] in contrast to artesunate with approximately 1 h [40]. Clinical trials testing mefloquine [41] and ACTs like artesunate-amodiaquine [42], artesunate-sulfalene–pyrimethamine, or artemether-lumefantrine [43,44] in patients concomitantly infected with *P. falciparum* and *Schistosoma spp*. reported a beneficial secondary antischistosomal efficacy, e.g. reaching an egg reduction rate of 98% for mefloquine. However, treatment trials specifically designed to determine ACT efficacy in schistosomiasis found less promising results [26]. In our first explorative analysis of data obtained from a non-randomized, observational antimalarial treatment trial done in Gabon, 3 out of 4 children who received pyronaridine-artesunate for treatment of uncomplicated malaria were cured from *S. haematobium* infections. We are aware that the infection intensity of the children was low making them more likely to be cured from a *Schistosoma* infection. Prospectively conducted randomized, controlled, blinded trials are needed to properly characterize the effect of pyronaridine-artesunate against (concomitant) *Schistosoma* infections. An important aspect is the investigation of drug activity against the juvenile worms, which requires a specifically designed experimental model. Such clinical work can relatively easily be done with compounds approved for use in humans and is a straightforward approach to conclude on the antischistosomal potential of pyronaridine (at least at the antimalarial dosage). From our work, we have evidence towards activity against young worms, but it is not yet clear if there is also a beneficial effect against adult worms. Despite a detrimental effect of pyronaridine on *in vitro* cultured *ex vivo* adult worms (assay read-out after 14 days of culture where 7 days drug exposure was followed by a 7 days drug washout phase), no inhibition of adult worms were observed in Schistosoma infected mice. Methylene blue is another candidate which warrants future clinical investigation of a concomitant beneficial effect on schistosomes but more preclinical data would be helpful for decision making.

Our results demonstrate promising antischistosomal properties of a selection of antimalarials, especially pyronaridine, which was highly active against juvenile *S. mansoni* worms, and methylene blue, which was active against adult *S. mansoni* worms. First hints from clinical data towards pyronaridine-artesunate activity against urinary schistosomiasis are reported. These results demonstrate the potential of antiplasmodial compounds, especially pyronaridine and methylene blue, as candidate compounds for the development of novel schistosomiasis therapies, or at least as lead compounds for even more active derivatives.

Methods

Ethics statement

Animal experiments were conducted in accordance with German laws after approval by the local authorities (Regierungspräsidium Tübingen, Germany, animal testing license T1/16).

The clinical study was approved by the institutional ethics committee of CERMEL (reference number: CEI- 006/20189) and was registered at ClinicalTrials.gov Identifier NCT03201770. The trial was conducted according to ICH-GCP and national guidelines. Written informed consent was obtained from the parents of the children.

Schistosoma life cycle

The life cycle of *S. mansoni* (Puerto Rico PR-1 strain) is routinely maintained in NMRI mice (female, 20–22 g) and in *Biomphalaria glabrata* snails. Mice were infected percutaneously by bathing the mice´ feet for 1 hour in cercaria containing waters (50 cercaria per mouse to keep the lifecycle or 100 cercaria for *in vivo* studies). To obtain schistosomula for *in vitro* drug testing, cercaria shed from infected snails were manually transformed. Adult worms were obtained from mice after 8–10 weeks of infection. All procedures were previously described [23].

Compounds

Artesunate, mefloquine hydrochloride, amodiaquine dihydrochloride dihydrate, chloroquine diphosphate, primaquine phosphate, quinine hemisulfate, atovaquone, methylene blue, pyronaridine tetraphosphate, doxycycline hyclate, clindamycin and praziquantel were purchased from Sigma-Aldrich. Ferroquine was obtained from Sanofi-Synthelabo, proguanil and cycloguanil from Jacobus Pharmaceutical Company, tigecycline from Wyeth, and mirincamycin hydrochloride enantiomers from Maldevco [45]. All compounds were dissolved in sterile DMSO except for quinine for which methanol was used and pure M199 medium (without additives) was used to dissolve proguanil, cycloguanil, clindamycin, and pyronaridine. The stock concentration was 50 mM for artesunate, amodiaquine, chloroquine, atovaquone, quinine, and primaquine, and 100 mM for praziquantel, proguanil, cycloguanil, methylene blue, pyronaridine, clindamycin, doxycycline, and mirincamycin enantiomers, respectively. Mefloquine was dissolved to 24 mM and ferroquine to 12.5 mM. All stocks were freshly prepared for the study and stored at -20˚C. Maximum concentration of the solvent (DMSO, methanol) in the *in vitro* assays did not exceed 0.8% and did not interfere with parasite viability.

In vitro drug sensitivity testing of schistosomula (step 1)

Compounds were pre-dosed (25 μl/well) in 96-well flat-bottom plates in 2-fold or 3-fold serial dilutions including wells with schistosomula culture medium only (SCM: phenol-red free medium 199 (Thermo Fisher Scientific), 1% decomplemented fetal bovine serum (FBS, Gibco), and 200 μg/mL streptomycin/200 U/l penicillin (Gibco)). The range of drug concentrations tested is given in S1 Table. Mature schistosomula (24 h old) in SCM were added to pre-dosed wells to obtain 100 schistosomula per well in a total volume of 225 μl/well. The exact number of schistosomula per well was counted by microscopy. Schistosomula drug assays were kept at 37˚C and 5% CO_2.

Viability of schistosomula per well was assessed by three different methodologies: i) assessment of morphology and motility by microscopy after 3 and 7 days of drug exposure, ii) resazurin assay, and iii) lactate assays, both done after 7 days of assay start. For the microscopic read-out, all schistosomula per well were visually judged using an inverted microscope (Nikon eclipse Ti 20x objective) for motility and morphology and classified either as viable (movement and normal appearance) or dead (severe morphological changes, e.g., granularity, blebbing, and/or no movement within 10 s of observing an individual worm)). Viability was expressed as % of total worms/well. The resazurin assay was done by removing 20 μl of supernatant/well and adding 20 μl AlamarBlue reagent (ThermoFisher Scientific cat. no. DAL1025). The plate

was incubated at 37˚C, 5% CO_2 and read in a fluorometer at 530 nm/590 nm after 24 h. Of the removed supernatant, 10 μl were further analyzed to quantify lactate levels as described earlier [23]. Briefly, fluorometric L-lactate assay kit (Abcam cat. no. ab65330) and 96-well, black-sided, optical clear-bottom plates (Corning cat. no. 3340) were used to quantify lactate levels following manufacturer´s specifications with minor modifications. After 40 min of incubation at room temperature, the plate was read by the fluorometer at 530 nm/590 nm. Both assays included SCM to determine the background resazurin and lactate levels, respectively, for normalization of readings of the experiment measurements.

Drug sensitivity testing of *ex vivo* adult worms (step 2)

For the *in vitro* drug sensitivity assays, *ex vivo* adult worms were set in 24 well-plates with 2 worms (1 pair or 1 separated pair, or 2 individual worms)/well in 1 ml adult worm culture medium (ACM; phenol-red free RPMI, 100 U/ml penicillin plus 100 μg/ml streptomycin, 5% FBS) and were kept at 37˚C and 5% CO_2. Worms Parasites were adapted to culture conditions for 24 h. Every experiment included 1 μM PZQ as positive control and 0.1% DMSO and ACM only as negative controls. Drugs were tested at concentrations of 1 μM, 5 μM, 10 μM, and 30 μM for 7 days, and 30 μM for 1 day. To control for irreversibility of drug effects, drug/medium mixture was replaced by fresh ACM (without drug) and *in vitro* culture was continued for an additional 7 days (drug wash-out). Viability of the worms was assessed by microscopy at the end of the drug wash-out period. Viability was categorized into 3 stages based on the worms´ motility (normal movement = viable, some/little movement = affected, no movement = dead) assessed via an inverted microscope (Wild Heerbrugg M7A Stereo Zoom microscope 6x-31x).

In vivo mouse studies (step 3)

Mice infected with *S. mansoni* at the juvenile life cycle stage (2 weeks after infection, 60 mice in groups of 6 animals) or at the adult stage (9 weeks after infection, 30 mice in groups of 6 animals) were treated with the study drugs dissolved in 7% Tween 80 and 3% ethanol in PBS and administered via oral gavage. Methylene blue (twice per day 50 mg/kg) and pyronaridine (500 mg/kg) were given for 5 consecutive days. Positive control drugs were artesunate (5 days 300 mg/kg/day) or praziquantel (once 300 mg/kg) for the juvenile and the adult life cycle stages, respectively and negative control was solvent (10 ml/kg). After 8 weeks or 2 weeks following treatment start, mice were sacrificed and recovered worms were counted to determine efficacy against juvenile or adult stages, respectively. Liver granulation was determined by counting the number of *S. mansoni* eggs on the outer surface of the liver and classified into no granulation or 0 eggs (score of 0), low granulation or 1–10 eggs (score of 1), medium granulation or 11–99 eggs (score of 2), and high granulation or $\geq$100 eggs (score of 3). Two independently conducted experiments were done for the juvenile stage as well as for the adult worms (for adult worms results from one experiment are shown, as infection rates of mice were not sufficient in the repeated experiment (shown in S4 Table).

The sample size (number of mice/group) was calculated based on an assumed 80% worm reduction and a variance of 30% in the group of untreated animals, thus a minimum of 6 animals were needed per group (treated vs. untreated, alpha = 0.05, beta = 0.2).

Pilot clinical study (step 4)

An ongoing observational study conducted in Lambaréné, Gabon, assessed the effect of the treatment of acute uncomplicated malaria with antimalarial combination therapy on concomitant urogenital schistosomiasis. Here we present data from pediatric patients treated with

pyronaridine-artesunate. Study participants received once-daily fixed dose pyronaridine-artesunate therapy (180 mg pyronaridine tetraphosphate plus 60 mg artesunate, Pyramax, Shin Poong Pharmaceutical Co. Ltd) for three days following label information (1 tablet for 20 to 23.9 kg, 2 tablets for 24 to 44.9 kg). Participants positive for *S. haematobium* eggs by urine filtration and microscopy before treatment start were included into the analysis. Urine was resampled 28 days later, and egg excretion was assessed.

Data analysis

The response of *in vitro* schistosomula assays was either expressed as % viability (microscopy data), or as normalized relative light units (RFU) for resazurin and lactate assays. Data were analyzed by GraphPad Prism 6 v6.07 to determine the 50% inhibitory concentrations (IC50) applying a four-parameter logistic regression analysis of log-transformed drug concentration response curves. After data collection, outliers were identified by ROUT and removed (Juvenile set: 2 data points, Adult set: 1 data point). Cleaned data were analyzed and median (IQR) values were calculated. Kruskal-Wallis multiple comparison was done to identify significant differences in worm counts between compound and control group (no drug). Dunn´s test was done to correct for multiple comparisons. Adjusted P-values are reported.

Supporting information

S1 Fig. Assay quality controls of *in vitro* drug assays with schistosomula (step 1). Individual results, median and IQR of schistosomula viability in medium only and in 0.8% DMSO after 7 days *in vitro* culture (negative controls), and IC50 of mefloquine (MQ, positive control), respectively. Every drug assay included these 3 controls.
(TIF)

S2 Fig. Agreement of microscopy and resazurin assay. Bland-Altman plot of difference of microscopy and resazurin assay based IC50 values obtained from schistosomula after 7 days of drug exposure. Analysis was done using individual in vitro assay outcomes of all compounds tested. Upper and lower dotted lines represent the 95% limits of agreement.
(TIF)

S3 Fig. *In vitro* drug assay of *ex vivo* adult *S. mansoni* worms. Worms were exposed to A) no drug (negative control), B) 1 μM praziquantel, C) 30 μM pyronaridine, and D) 30 μM methylene blue, respectively, for 7 days followed by an additional 7 days without drug (drug washout, but medium only) to confirm the detrimental drug effect. Then viability was assessed (see photos A, B, C, and D and S1 Videos).
(TIF)

S4 Fig. Liver granulation after drug treatment of *S. mansoni*-infected mice. Mice were infected with 100 cercariae of *S. mansoni*. 14 days later (when the parasite is still in at a juvenile stage), mice were exposed to A: no drug (negative control), B: artesunate, C: pyronaridine, and D: methylene blue. Mice were euthanized (CO_2-inhalation) eight weeks post treatment and liver were taken out later to document the burden of eggs (granulation).
(TIF)

S5 Fig. Dose-response curves of *in vitro* schistosomula assays (step 1) resulting from different drugs and assay read-outs. Dose-response curves of viability assessment obtained by microscopy at 72 h and at day 7, resazurin assay at day 7, and lactate assay at day 7 are shown for mefloquine, pyronaridine, and tigecycline. Every drug was measured independently three times (red, yellow, and green lines). The 50% inhibitory concentration (IC50) per drug per

assay is calculated from curves following a sigmoidal dose-response, see mefloquine all assays. Pyronaridine effectively inhibited schistosomes when evaluated by microscopy. Despite a dose-dependent inhibition of the worms as shown by resazurin and lactate assays (experiments D2 and D4), the response was not sigmoidal and thus an IC50 could not be derived. Tigecycline is an example a drug not active against schistosomula.
(TIF)

S1 Table. Test compounds. Drug concentration range (in μM) and dilution factor (DF) are indicated for *in vitro* drug testing against schistosomula (step 1). Each series of drug concentration tested included always a well without any drug (medium only). PC: positive control drug, Abb.: abbreviation, i.d.: in development.
(PDF)

S2 Table. Published data on antimalarials that report drug activity against *S. mansoni*. All data are expressed in molarity and were converted (*) if necessary. WBR Worm burden reduction, LD: lethal dose, d: days, n.d.: no data was found in the literature.
(PDF)

S3 Table. Baseline characteristics of study participants. The children had the following egg counts: * 4, 6, or 14 eggs/10 ml urine. #: 256, 322, or 2032 eggs/10 ml urine. Infection intensity: light $\leq$ 50 eggs/10 ml urine, heavy $>$ 50 eggs/10 ml urine. By Combur10 test: microhematuria: $>$5 ery/μl, proteinuria: $>$ 0.3 g/l, leukocyturia: $>$ 10 leu/μl.
(PDF)

S4 Table. Activity against adult *S. mansoni* in mice (low infection rates). Mice infected with *S. mansoni* for 9 weeks (worms are in the adult stage) were treated with methylene blue (MB) or pyronaridine (PY). Negative control treatment: No drug and solvent, positive control treatment: Praziquantel (PZQ). WBR is referred to the "No drug" group. WBR: Worm burden reduction, LGS: Liver granulation score. * 1 mouse died during the treatment week. [a] Interquartile range: IQR. Results from one experiment are shown. Of note: These are results of the repeated experiment, but as infection rates in control mice were low, they are presented here separately.
(PDF)

S1 Data. Excel spreadsheet with underlaying numerical data. Data for figures are organized in tabs.
(XLSX)

S1 Videos. *Ex vivo* adult *S. mansoni* worms after drug exposure. Worms were exposed to S3A) no drug (negative control), S3B) 1 μM praziquantel, S3C) 30 μM pyronaridine, and S3D) 30 μM methylene blue, respectively, for 7 days followed by an additional 7 days without drug (drug wash-out, but medium only).
(ZIP)

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3. Discussion:

Malaria and schistosomiasis cause significant morbidity and mortality and are often found together in the same geographical location. The development of vaccines and novel drugs is needed to decrease morbidity and mortality associated with both of these diseases. Vaccines can prevent infection and/or diseases by preparing an individual's immune system towards a pathogen-specific antigen, but drugs are highly important to treat acute diseases. This work describes the *in vitro* and *in vivo* drug activity of antibiotics, epigenetic regulators, and compounds targeting the hemozoin synthesis pathway against *P. falciparum* and/or *S. mansoni* with insights on the biological processes targeted by these drug classes.

3.1 Tetracyclines

Antibiotics act against bacteria and/or organelles in eukaryotes of prokaryotic descent, by inhibiting important cellular processes vital to the bacteria's/eukaryote's survival. One of the first antibiotics, penicillin, was discovered in 1928 and implemented for the treatment of gonococcal infections [98]. Following this discovery, many other antibiotics were developed in the mid-1900s including the class of tetracycline antibiotics, which were known for their broad-spectrum antibacterial activity against microorganisms such as Gram-positive and Gram-negative bacteria, chlamydiae, mycoplasmas, and protozoan parasites [40]. Antibiotics of the tetracycline class were the first antibiotics to be used for the treatment of *P. falciparum* and *P. vivax* malaria [80]. Doxycycline, a synthetic tetracycline, was officially recommended for chemoprophylaxis of *P. falciparum* malaria by WHO in 1985, due to the emergence and spread of chloroquine resistance [80]. Today, doxycycline is often combined with quinine in treatment therapies and used for chemoprophylaxis in multidrug resistance areas, including Southeast Asia [80]. So far, doxycycline and other tetracyclines, including tigecycline, and minocycline, have been shown to inhibit *P. falciparum in vitro* and *in vivo* [99]. Moreover, a recently FDA-approved synthetic tetracycline and derivative of tigecycline, called eravacycline, was developed to overcome classical resistance mechanisms developed by bacteria against tetracyclines and was approved for the treatment of complicated intra-abdominal infections [100].

Eravacycline has a similar structure to tigecycline with two changes to the D-ring of its tetracycline core, including the replacements of a fluorine atom at the dimethylamine moiety at C-7 and a pyrrolidinoacetamido group at the 2-tertiary-butyl glycylamido at C-9 [101]. Moreover, eravacycline is available as an intravenous formulation, similar to tigecycline, since the oral formulation has low bioavailability after intake at approximately 28% [101–103]. In this work, eravacycline was assessed for its *in vitro* drug activity against *P. falciparum* clinical isolates collected in Gabon, compared to doxycycline, tetracycline, and tigecycline, and the apicoplast was evaluated as a potential drug target. The additional assessment of drug candidates *in vitro* against clinical *P. falciparum* isolates can inform about cross-resistances of parasite strains circulating in malaria endemic areas [104].

Eravacycline showed high activity (14nM) against the culture-adapted *P. falciparum* strain 3D7 and clinical isolates in a 6-day assay. Compared to the *in vitro* activity of other tetracyclines, eravacycline was more active than tigecycline (38nM) and doxycycline (241nM) after prolonged drug exposure (6-day assay) against the 3D7 laboratory strain. Propagation of the laboratory strain parasites using the 3-day assay occurred after incubation with all of the antibiotics, though, most compounds showed some drug activity, especially eravacycline with the highest. In clinical isolates, the delayed death effect for eravacycline was less pronounced, but inhibited parasite propagation during the first cycle. The relatively high activity of eravacycline on the first-generation parasites, within the first cycle, may suggest an additional effect on other organelles, which may include the mitochondria, though this was not evaluated in this work, but may be beneficial to evaluate in future studies. Delayed parasite death in the second generation, following eravacycline treatment and re-established viability after IPP supplementation, strongly indicates the apicoplast to be the target organelle, thereby resulting in the inhibition of isoprenoid precursor biosynthesis.

Compared to the other tetracyclines, eravacycline was more active against asexual blood stage *P. falciparum* parasites. These results show the potential of novel antibiotics as candidate drugs for antimalarial therapy. Currently, ACTs are the recommended first-line treatment for uncomplicated *falciparum* malaria and the first-line treatment of patients

with severe malaria is intravenous artesunate, though intramuscular artemether or intravenous quinine may be used if intravenous artesunate is not available [105]. Patients suffering from severe malaria often experience symptoms similar to those of other acute bacterial or viral infections [106], which makes diagnosis of severe malaria difficult in resource-limited health care settings. New combination therapies can be developed to include an antibiotic to not only treat malaria, but also bacterial co-infections [107,108]. Doxycycline is currently the only recommended drug of the tetracycline class for prophylaxis and the treatment of malaria, though it is not recommended for pregnant women and children younger than eight years of age [79], and is also indicated in the treatment of cholera, Lyme disease, Rickettsia, and typhus fever [109]. Doxycycline is a slow-acting blood schizonticide that achieves good treatment efficacy when given together with rapidly acting antimalarials, which is why it is not employed as a monotherapy for *P. falciparum* infections [80]. Moreover, doxycycline has limited activity on the liver stages of *Plasmodium,* which indicates it is not effective against *P. vivax* hypnozoites, and must be taken for 3-4 weeks post malaria exposure to suppress parasites that may emerge from the liver [80]. Eravacycline was shown to be more active *in vitro* than other tetracyclines by targeting the apicoplast and may inhibit parasites more quickly than doxycycline.

In protozoan parasites, such as *P. falciparum,* many antibiotics are known to act against the apicoplast, an organelle of prokaryotic descent, with the essential role of isoprenoid precursor biosynthesis, vital for parasite survival [110]. This makes the apicoplast an interesting target for the development of new antimalarials, since human cells do not contain this organelle. For example, doxycycline, like other tetracyclines, acts by inhibiting the smaller 30S ribosomal subunit within the apicoplast, thereby blocking the attachment of the transfer RNA-amino acid to the ribosome [111]. Compounds that inhibit the apicoplast cause a typical “delayed death” effect in *Plasmodium* parasites, whereby the first-generation parasites are not affected, but the second generation dies, even in the absence of the drug [81].

Before the apicoplast was considered the target of the tetracyclines, the mitochondrion was evaluated as a potential candidate [112]. Several studies suggest that tetracyclines may inhibit mitochondrial protein synthesis, such as minocycline, which has

been shown to affect the transcription of genes encoded by the mitochondrion, more so than other tetracyclines, due to its high lipophilicity [113]. Similarly, other researchers suggest that the concentration of tetracyclines reached inside the mitochondria is insufficient to deplete respiratory chain enzymes [40]. Compounds with similar features to minocycline may be able to increase mitochondrial membrane permeability, thereby increasing the concentration of the drug inside the mitochondrion, ultimately inhibiting protein synthesis, and killing the parasite. Further studies evaluating the activity of eravacycline against the mitochondrion in *P. falciparum*, would be beneficial to elucidate whether or not this organelle is also a target.

Little is reported in regard to drug activity of antibiotics against the *Schistosoma* parasites and most of those that have been tested are shown to be inactive. However, the apicoplast may not be the only organelle of procaryotic descent suitable as a drug target candidate for antibiotics. The mitochondrion also plays an important role in the malaria parasite's life cycle and may be a useful drug target, not only against *Plasmodium* parasites, but *Schistosoma* parasites as well [31]. This, however, is only sensible if the human mitochondrion is not also affected by these drugs, thereby leading to toxicity in human cells. A key function of the mitochondrion in *P. falciparum* is to provide orotate for pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase [114]. Atovaquone, a quinone, used in combination with proguanil (Malarone) for the treatment of children and adults with uncomplicated malaria or for chemoprophylaxis, inhibits mitochondrial electron transport at the bc_1 complex, thereby affecting the concentration of metabolites in the pyrimidine biosynthetic pathway [115]. Moreover, atovaquone does not inhibit the human mitochondrial bc_1 complex, making it an ideal antimalarial drug [116]. In our work, the insensitivity of schistosomes to antibiotics, including doxycycline, was confirmed although some noteworthy antischistosomal effects were seen for both isomers of mirincamycin [78]. Furthermore, atovaquone was shown to be inactive in our work, though studies have found it to be a highly active inhibitor (nanomolar range) of the dihydroorotate dehydrogenase enzyme of *S. mansoni* [117,118], when it was recombinantly expressed. This may occur based on the difference in *S. mansoni* strains or *in vitro* assays used. Though tigecycline was inactive, based on day 7 microscopy data, the minimal activity of mirincamycin may further support the evaluation of eravacycline as

a potential antischistosomal compound. Eravacycline should be further evaluated for its antiplasmodial activity *in vivo*, including the potential off-target activity against the mitochondria, and considered for evaluation against other parasites including *Schistosoma*.

3.2. Histone deacetylase inhibitors

Histone acetylase enzymes play an important role in epigenetic and non-epigenetic regulation by increasing acetylation of histones, which causes the chromatin to relax, thereby increasing gene transcription [119]. Conversely, histone deacetylase enzymes initiate deacetylation of histones, thereby causing chromatin condensation, which decreases gene transcription [119]. To date, 18 human HDACs have been identified and are divided into four major classes, including class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9, 10), class III (SIRTs 1–7) and class IV (HDAC 11)) [120]. Moreover, non-histone proteins, including chaperones, signaling mediators, cell motility proteins, may also be affected by acetylation, which can indirectly increase or repress gene transcription [121]. HDAC6, a cytoplasmic deacetylase, predominantly deacetylases non-histone substrates, which play an important role in cell motility [122,123]. One example is alpha-tubulin, which is one of the most widely studied non-histone HDAC6 substrates and is associated with the regulation of microtubules (important for cytoplasmic transport of membrane vesicles and cell division) [122]. Overexpression of alpha-tubulin can cause it to accumulate within the cell, thereby leading to cell division arrest [124]. Moreover, HDAC6 also associates with cortactin, a class II nucleation promoting factor, which binds to the actin cytoskeleton and influences its stability [123]. When cortactin is hyperacetylated, cell motility can be impaired [123]. Another substrate of HDAC6 includes the heat shock protein (Hsp90), which functions as a molecular chaperone and plays an essential role in cell division and signaling pathways [125]. These examples show the significance of non-histone HDAC6 substrates in regard to important cellular processes, thereby making HDAC6 inhibitors interesting compounds for the development of novel drugs.

Histone deacetylase inhibitors (HDACi) are compounds that inhibit histone deacetylase enzymes or associated non-histone proteins, thereby enhancing acetylation

and upsetting important cellular functions. In humans, HDACi have been primarily implemented for the treatment of various cancers including B-cell lymphoma (abexinostat), multiple myeloma (quisinostat), prostate cancer (practinostat), breast cancer (entinostat), and pancreatic cancer (tacedinaline) [126–128]. HDACi are also being investigated against human parasitic diseases such as malaria, schistosomiasis, trypanosomiasis, toxoplasmosis, and leishmaniasis [88]. In *P. falciparum*, six HDACs homologous to human HDACs have been identified and could be explored for the development of novel inhibitors [129]. So far, broad-spectrum HDACi have primarily been investigated as potential antiplasmodial drugs, also known as pan-HDAC inhibitors, which target multiple HDACs. Apicidin, a fungal metabolite and one of the first HDACi evaluated against *Plasmodium* parasites, was found to be a highly active pan-HDAC inhibitor targeting mainly class I HDACs [89]. Other clinically approved HDACi, including vorinostat, romidepsin, belinostat, and panobinostat affect the growth of various *Plasmodium* spp., including drug resistant *P. falciparum* strains [130]. Though, many of these inhibitors have been shown to also be toxic to human cells, therefore limiting their use in the clinic [90]. However, some HDACi are known to be less harmful to mammalian cells, including selective inhibitors of human HDAC6 class II enzymes, supported by evidence from knock-out mice [131], that can be further evaluated as potential antiplasmodial candidates, including in clinical studies [132].

In the second study, the *in vitro* potency of novel HDAC6-inhibitors against *P. falciparum* was evaluated. Selective inhibitors of human HDAC6 (hHDAC6) have been shown to exert lower levels of cytotoxicity to human cells compared to HDAC class I inhibitors [133]. In this work, 12 newly developed peptoid-based HDACi candidates were tested against *P. falciparum* [91,132]. Our results showed candidate 1u, N-(2-(Cyclohexylamino)-2-oxoethyl)-N-(4-(hydroxycarbamoyl)benzyl)-4-isopropylbenzamide, to be the most potent HDACi against *P. falciparum* laboratory strains and clinical isolates. 1u was obtained by replacing the N, N-dimethylamino group from molecule 1h with a less polar isopropyl group [132]. Additionally, compared to other HDACi, 1u was also shown to be toxic to mammalian HEPG2 cells at approximately 10 µM, but had high parasite selectivity (>1000-4000) [132]. Normally, an LC50<10–50 µM is used to categorize pharmaceutical compounds, thus classifying 1u as borderline hepatotoxic [134].

Moreover, the stability of 1u was shown to vary, which could be seen in the IC50 ranges in the data [104]. 1u is a potential lead compound for further development as an antimalarial chemotherapeutic with promising selectivity towards *Plasmodium* parasites, but requires improvement of chemical stability and decreased toxicity to human cells.

HDACi have the potential to be further developed against other parasitic diseases, including schistosomiasis. Class I HDACs in *S. mansoni* (SmHDAC1, 3, and 8) have been identified and studied, which could make interesting therapeutic targets in the development of novel HDACi. For example, smHDAC8 is highly expressed in *S. mansoni,* but not so much in humans when compared to other HDACs, and is structurally different [135]. SmHDAC8 is shown to be involved in cytoskeleton organization due to its association with actin proteins [136]. Moreover, smHDAC8 knocked down mice had a decreased worm and egg burden [137]. *In vitro* screenings reported drug activity of HDACi targeting smHDAC8 to be in the low micromolar range [135]. Not much has been reported on the toxicity in human cells of HDACi targeting class I smHDACs. However, class II enzymes show lower similarity to human homologues, which would include smHDAC6, thereby making them potentially less toxic than other smHDACi [138]. Class II SmHDACs, including smHDAC6, should be evaluated for their role in *S. mansoni* and novel HDACi should be investigated as potential lead compounds for further development as antimalarial chemotherapeutics.

3.3. Antiplasmodial compounds

The only drug available for the treatment of schistosomiasis is praziquantel and acts primarily against the adult life cycle stage. In the prepatent period, schistosomes of up to approximately eight weeks are not affected, which limits the control and elimination efforts of schistosomiasis by praziquantel mass drug administration programs. Drugs targeting the adult and prepatent life cycle stages of *Schistosoma* parasites would be more effective in treating patients. Existing antimalarials may be repurposed to target the prepatent stages of *Schistosoma* and treat patients with co-infections. Some antimalarials are known to be active against both parasites, since certain biological mechanisms are similar, including the hemozoin synthesis pathway.

Plasmodium and *Schistosoma* parasites consume the host's hemoglobin, thereby forming hemozoin crystals as a byproduct. Hemozoin, also known as "malaria pigment," is crystalized heme and can be identified as the brown pigment seen in unstained malaria parasites or female schistosome adult worms. High quantities of toxic free heme are released from the digestion of blood, which can accumulate and can kill the parasites if left unchanged. Both parasites have developed intracellular detoxification mechanisms to rapidly convert toxic free heme into hemozoin, an inert form [77]. Since this biological process is vital to the parasite's survival and is not found in humans, it is an appealing target for the development of novel drugs. Drugs of the quinoline class, for example, are known to kill the parasites by targeting the hemozoin synthesis pathway [139]. Chloroquine, prevents biocrystallization of heme by forming the highly toxic FP-chloroquine complex, thereby leading to its accumulation in the cell and killing the parasite [140]. Pyronaridine, the partner drug of artesunate in the ACT Pyramax, inhibits ß-hematin formation, thereby causing toxic hematin accumulation in the digestive vacuole of the malaria parasite [141]. Methylene blue, a dye and medication used to treat methemoglobinemia, is also a strong inhibitor of hemozoin crystal growth in *P. falciparum* [142] and is being investigated as an antimalarial *in vivo* in human clinical trials [143]. Pyronaridine has been investigated against adult schistosomes with little success, but pyronaridine and methylene blue may be active against the prepatent life cycle stages of *Schistosoma* parasites.

In the final study, we investigated antimalarials as possible drug candidates, especially pyronaridine and methylene blue, for alternative treatment strategies of *Schistosoma* infections, especially for the juvenile stages. A total of 16 compounds with known schistosome activity were investigated plus mefloquine and artesunate, which were used as controls. *In vitro* activity against the schistosomula stages confirmed the drug activity of mefloquine, artesunate, chloroquine, and ferroquine [78]. Moreover, pyronaridine and methylene blue rapidly inhibited the juvenile stages and *ex vivo* adult worms. Pyronaridine and methylene blue were further tested *in vivo* against mice infected with *S. mansoni* at the juvenile stage (two weeks after infection) or adult stage (nine weeks after infection). Pyronaridine was found to be active against the juvenile forms with little to no activity against adult worms *in vivo*, similar to data published by Keiser et al. [54], which

may suggest an inadequate amount of bioavailability of the drug to kill adult worms *in vivo*. Surprisingly, dissection of the mice treated with pyronaridine against the juvenile life cycle forms revealed no eggs in the livers, thereby confirming high efficacy against prepatent life cycle stages. On the contrary, methylene blue, was potent against the adult worms *in vivo* in mice, but not the juvenile stage. Pyronaridine and methylene blue are appealing candidates for further development as novel antischistosomal compounds since they play a role in the inhibition of the hemozoin synthesis pathway.

Furthermore, pyronaridine-artesunate (Pyramax) was investigated in a small pilot clinical study for its drug activity *in vivo* against uncomplicated malaria and urogenital schistosomiasis. 3 out of 4 children who received Pyramax for the treatment of uncomplicated malaria were cured from *S. haematobium* infections. Moreover, Pyramax reduced *S. haematobium* egg excretion from 10/10 ml urine to 0/10 ml urine. This work further provides evidence of the antischistosomal effect of pyronaridine *in vivo,* thereby raising the importance of further investigation of this drug against the *Schistosoma* parasite. Pyramax, one of the more recently available ACTs for the treatment of uncomplicated malaria, may also exhibit antischistosomal activity, since pyronaridine is known to target the hemozoin forming pathway in both parasites. To summarize, antiplasmodial compounds targeting the hemozoin synthesis pathway, including pyronaridine and methylene blue, may also be promising antischistosomal drug candidates for further development and investigating in human clinical trials.

3.4. Conclusion

Parasitic diseases, such as malaria and schistosomiasis, remain a leading cause of morbidity, disability, and mortality in tropical and subtropical regions of the world. The number of available drugs to treat these diseases effectively is limited. ACTs for the treatment of malaria are met with parasite drug resistance, primarily in Southeast Asia, and praziquantel is the only efficient drug available for the treatment of schistosomiasis. Thus, new medications are needed to effectively treat individuals infected by both parasites and to control for the possible spread and development of resistance. Moreover, *Plasmodium* and *Schistosoma* parasites are not only endemic in the same geographic range, but share similar intracellular biological mechanisms ideal for the identification of new targets and the development of novel chemotherapeutics against both parasites. Antibiotics, including those of the tetracycline class, are antimalarials known to target the apicoplast within *Plasmodium* parasites. Though most antibiotics have proven unsuccessful against schistosomes, the parasite's mitochondrion may be a useful target for the development of other compounds. Moreover, HDACs are important epigenetic and non-epigenetic regulators and have been identified in both parasites, thereby making these targets ideal for novel antimalarial and antischistosomal compounds. Lastly, antimalarials targeting the hemozoin synthesis pathway may also be useful against the juvenile stages of the schistosome parasite. This work sheds light on the drug activity of novel antimalarial and antischistosomal compounds and gives a brief overview of the biological processes inhibited in both parasites, which can be further investigated as novel drug targets.

4. Summary:

Parasitic diseases continue to be a leading cause of disability, morbidity, and mortality in tropical and subtropical regions of the world. Malaria, an acute and life-threatening disease, causing significant mortality, and schistosomiasis, a predominantly chronic disease leading to long-term morbidity, are often found together in the same geographical area, principally affecting people of low socioeconomic status. Artemisinin-based combination therapies are currently recommended by WHO for the treatment of patients with uncomplicated malaria. Recent reports from Southeast Asia show a delay in the clearance of malaria parasites from the bloodstream after treatment with artemisinin-based combination therapies. Thus, raising concerns that the parasites may spread from Southeast Asia to sub-Saharan Africa or occur *de novo*. Moreover, praziquantel is the only available drug for the treatment of schistosomiasis. However, this monotherapy presents with several challenges including that it is not active against juvenile worms and the cure rate is rarely 100%. A drug or drug combinations targeting the adult and prepatent life cycle stages of schistosomes would be more effective in treating patients and would significantly reduce pathogen transmission rates. The aim of this study was to assess the activity of compounds targeting organelles of prokaryotic descent, epigenetic regulators, and the hemozoin synthesis pathway in *Plasmodium* and *Schistosoma* parasites.

The first study evaluated antibiotics for their drug activity against *P. falciparum* and *S. mansoni*. The novel synthetic halogenated tetracycline derivative eravacycline, together with tetracycline, tigecycline, doxycycline, and the lincosamide clindamycin, were evaluated for their drug activity against *P. falciparum* with the apicoplast investigated as a potential target. Moreover, clindamycin, doxycycline, mirincamycin, and tigecycline were tested against *S. mansoni* schistosomula *in vitro*. Eravacycline showed the highest activity of all the tetracyclines in the 3-day and 6-day assays against *P. falciparum* clinical isolates in Gabon. Antibiotics tested against *S. mansoni* were shown to be inactive in our study, though mirincamycin exhibited minimal activity. These findings show the potential of novel antibiotics, especially eravacycline, as candidate antimalarial therapies and interventions in concomitant infections.

In the second study, the *in vitro* potency of novel HDAC-inhibitors was evaluated against *P. falciparum* clinical isolates collected in Lambaréné, Gabon and surrounding area. The potency of 12 peptoid-based HDAC-inhibitors of human HDAC6 against asexual stages of *P. falciparum* clinical isolates was investigated. All HDAC-inhibitors demonstrated 50% inhibitory concentrations at nanomolar ranges. Peptoid-based HDAC6-inhibitors should be lead structures further evaluated for the development of antimalarial chemotherapeutics and to further investigate epigenetic regulators, including HDACs, as targets in *Schistosoma* parasites.

In the final study, 16 antiplasmodial compounds were evaluated against *S. mansoni*, for alternative treatment strategies of *Schistosoma* infections. Pyronaridine, methylene blue, and 5 other antiplasmodial compounds were highly active *in vitro* against the larval stage schistosomula with IC50s below 10µM. Mice infected with *S. mansoni* at the prepatent stage were treated with pyronaridine which reduced the worm burden by 82% and cured 7 out of 12 animals, though the adult stages remained viable. Though, on the contrary, methylene blue inhibited adult worms by 60%, but did not cure the mice. As part of an observational pilot study in Gabon, children with *S. haematobium* infections were treated with pyronaridine-artesunate (Pyramax), resulting in a reduction of eggs excreted in urine from 10/10 ml to 0/10 ml, and 3 out of 4 children were cured. Pyronaridine and methylene blue should be further investigated as candidates in humans in clinical studies for schistosomiasis treatment, since both are already approved for human use.

This thesis provides innovative data on novel inhibitors targeting organelles of prokaryotic descent, epigenetic regulators, and the hemozoin synthesis pathway in *Plasmodium* and *Schistosoma* parasites. These findings will contribute to further innovation of novel inhibitors used to increase control and elimination efforts for both parasitic diseases in endemic areas. Prospective work will further evaluate novel compounds in clinical studies in humans and potentially provide more information on the targets of each of these drug classes.

5. German Summary:

Parasitäre Erkrankungen sind nach wie vor eine der Hauptursachen für die hohe Morbidität und Mortalität in tropischen und subtropischen Regionen weltweit. Malaria, eine lebensbedrohliche Infektionskrankheit, die eine hohe Sterblichkeitsrate verursacht, sowie die Bilharziose, eine überwiegend chronische Erkrankung, treten oft zusammen in denselben geografischen Gebieten auf und betreffen hauptsächlich Menschen mit niedrigem sozioökonomischem Status. Die WHO empfiehlt derzeit Artemisinin-basierte Kombinationstherapien für die Behandlung von Patienten mit unkomplizierter Malaria. Aktuelle Berichte aus Südostasien zeigen jedoch eine Verzögerung in der Beseitigung der Malariaparasiten aus dem Blutkreislauf nach der Behandlung mit Artemisinin- basierten Kombinationstherapien. Dies gibt Anlass zur Sorge, dass sich diese Parasiten von Südostasien nach Afrika ausbreiten oder dort neu auftreten könnten. Darüber hinaus ist Praziquantel das einzige verfügbare Medikament zur Behandlung der Bilharziose. Diese Monotherapie birgt jedoch mehrere Risiken. Unter anderem wirkt sie nicht gegen juvenile Würmer und die Heilungsrate beträgt selten 100 %. Ein Arzneimittel oder Arzneimittelkombinationen, die auf die Lebenszyklusstadien von adulten Schistosomen sowie die Präpatenzstadien abzielen, wäre bei der Behandlung von Patienten wirksamer und würde die Übertragungsraten von Krankheitserregern signifikant verringern. Das Ziel dieser Studie war es, die Aktivität von Wirkstoffen zu ermitteln, welche auf Organelle prokaryotischer Abstammung, epigenetische Regulatoren und den Hämozoin-Syntheseweg in Plasmodium- und Schistosoma-Parasiten abzielen.

In der ersten Studie der Dissertation wurden Antibiotika auf ihre Aktivität gegen *P. falciparum* und *S. mansoni* untersucht. Das neuartige, synthetisch halogenierte Tetracyclin-Derivat Eravacyclin wurde zusammen mit Tetracyclin, Tigecyclin, Doxycyclin und dem Lincosamid Clindamycin auf seine Aktivität gegen *P. falciparum* untersucht, wobei der Apicoplast als potenzielles Ziel untersucht wurde. Darüber hinaus wurden Clindamycin, Doxycyclin, Mirincamycin und Tigecyclin in vitro gegen *S. mansoni* Schistosomula getestet. Eravacyclin zeigte die größte Wirkung aller Tetracycline in den 3- und 6-Tages-Assays gegen klinische Isolate von *P. falciparum* aus Gabun. Die gegen *S. mansoni* getesteten Antibiotika erwiesen sich in unserer Studie als inaktiv, wobei

Mirincamycin eine minimale Aktivität aufwies. Diese Ergebnisse zeigen das Potenzial neuer Antibiotika, insbesondere von Eravacyclin, als potenzielles Therapeutikum gegen Malaria sowie andere Begleitinfektionen.

Im zweiten Teil der Dissertation wurde die *in vitro* Potenz neuartiger HDAC-Inhibitoren anhand klinischer *P. falciparum* Isolate aus Lambaréné, Gabun und Umgebung untersucht. Es wurde die Wirkung von 12 Peptoid-basierten HDAC-Inhibitoren von humanem HDAC6 gegen asexuelle Stadien von klinischen *P. falciparum* Isolaten untersucht. Alle HDAC-Inhibitoren zeigten IC50 Werte im nanomolaren Bereich. Daher sollten Peptoid-basierte HDAC6-Inhibitoren als Leitstrukturen für die Entwicklung weiterer Chemotherapeutika gegen Malaria dienen. Außerdem sollten sie zur weiteren Erforschung epigenetischer Regulatoren, einschließlich HDACs, als Zielstrukturen in Schistosoma-Parasiten in Betracht gezogen werden.

Im abschließenden Teil der Dissertation wurden 16 antiplasmodiale Substanzen gegen *S. mansoni* untersucht, um alternative Behandlungsstrategien gegen Bilharziose-Infektionen zu ermitteln. Pyronaridin, Methylenblau und fünf weitere antiplasmodiale Substanzen zeigten eine hohe *in vitro* Aktivität gegen das Larvenstadium einer Bilharziose-Infektion mit IC50-Werten unter 10 µM. Mäuse, welche mit *S. mansoni* im Präpatenzstadium infiziert und anschließend mit Pyronaridin behandelt wurden, zeigten eine Reduzierung der Wurmlast um 82%. 7 von 12 Tieren wurden geheilt, jedoch blieben die adulten Stadien von *S. mansoni* lebensfähig. Im Gegensatz dazu reduzierte Methylenblau die Lebensfähigkeit der erwachsenen Würmer um 60 %, eine Heilung konnte jedoch nicht erzielt werden. Im Rahmen einer Beobachtungs-Pilotstudie in Gabun wurden Kinder mit *S. haematobium* Infektionen mit dem Malariamedikament Pyronaridin-Artesunat (Pyramax) behandelt. Dies führte zu einem Rückgang der im Urin ausgeschiedenen Eier von 10/10 ml auf 0/10 ml Urin und 3 von 4 Kindern wurden geheilt. Pyronaridin und Methylenblau sollten für die Behandlung der Bilharziose weiter klinischen

Studien am Menschen untersucht werden, da beide für die Anwendung am Menschen zugelassen sind.

Diese Dissertation liefert innovative Daten zu neuartigen Inhibitoren, welche auf Organelle prokaryotischer Abstammung, epigenetische Regulatoren und den Hämozoin-Syntheseweg in Plasmodium- und Schistosoma-Parasiten wirken. Diese Erkenntnisse werden zu weiteren Innovationen neuartiger Inhibitoren beitragen, die zur verstärkten Bekämpfung und Eliminierung beider parasitärer Erkrankungen in endemischen Gebieten eingesetzt werden können. Zukünftige Arbeiten werden neue Substanzen in klinischen Studien am Menschen untersuchen und möglicherweise weitere Informationen über therapeutische Ansatzpunkte dieser Wirkstoffklassen liefern.

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7. Declaration of contributions

The doctoral dissertation entitled “Assessment of the drug activity of anti-infectives against *Plasmodium falciparum* and *Schistosoma mansoni*” is based on three publications, accomplished by Erik Koehne as a first author, and form the backbone of this dissertation. We declare that Erik Koehne contributed substantially to all three manuscripts as follows:

Publication 1: Koehne E, Kreidenweiss A, Bayode RA, Zoleko Manego R, McCall MBB, Mombo-Ngoma G, Adegnika AA, Agnandji ST, Mordmüller B, Held J. *In vitro* activity of eravacycline, a novel synthetic halogenated tetracycline, against the malaria parasite *Plasmodium falciparum.* J Glob Antimicrob Resist. 241:93-97. March 2021.

Erik Koehne and Jana Held managed the study concept, design, and acquisition of data. Data analysis, interpretation of data, and drafting of the manuscript were completed by Erik Koehne, Jana Held, and Andrea Kreidenweiss. Technical or material support was given by Erik Koehne, Jana Held, Andrea Kreidenweiss, Bayode Romeo Adegbite, Rella Zoleko Manego, Matthew McCall, Ghyslain Mombo-Ngoma, Ayola Akim Adegnika, Sélidji Todagbé Agnandji, and Benjamin Mordmüller. All authors reviewed and approved the final manuscript.

Publication 2: Koehne E, Kreidenweiss A, Zoleko Manego R, McCall MBB, Mombo-Ngoma G, Mackwitz MKW, Hansen FK, Held J. Histone deacetylase inhibitors with high *in vitro* activities against *Plasmodium falciparum* isolates collected from Gabonese children and adults. Sci Rep. 9(1):17336. November 2019.

Erik Koehne and Jana Held managed the study concept, design, and acquisition of data. Data analysis, interpretation of data, and drafting of the manuscript were completed by Erik Koehne, Jana Held, and Andrea Kreidenweiss. Technical or material support was given by Erik Koehne, Jana Held, Andrea Kreidenweiss, Bayode Romeo Adegbite, Rella Zoleko Manego, Matthew McCall, Ghyslain Mombo-Ngoma, Marcel Karl Walter Mackwitz, and Finn Hansen. All authors reviewed and approved the final manuscript.

Publication 3: Koehne E, Zander N, Rodi M, Held J, Hoffmann W, Zoleko Manego R, Ramharter M, Mombo Ngoma G, Kremsner PG, Kreidenweiss A. Evidence for *in vitro* and *in vivo* activity of the antimalarial pyronaridine against *Schistosoma*. Plos NTD. June 2021. 15(6):e0009511. June 2021.

Erik Koehne and Andrea Kreidenweiss managed the methodology, interpretation of the results, and drafting of the manuscript. Data acquisition, data analysis, and interpretation of data were completed by Erik Koehne, Andrea Kreidenweiss, and Nina Zander. The recruitment of patients was managed by Rella Zoleko Manego, Ghyslain Mombo-Ngoma, and Michael Ramharter. Technical or material support was given by Erik Koehne, Andrea Kreidenweiss, Nina Zander, Miriam Rodi, Wolfgang Hoffmann, Rella Zoleko Manego, Michael Ramharter, Ghyslain Mombo-Ngoma, and Peter G. Kremsner. All authors reviewed and approved the final manuscript.

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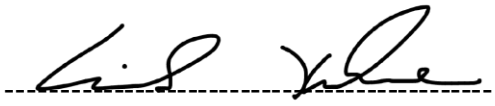

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