Antimalarial Activity of the Myxobacterial Macrolide Chlorotonil A

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Abstract

Myxobacteria are gram negative soil-dwelling bacteria belonging to the phylum proteobacteria. They are a rich source of promising compounds for clinical application such as epothilones for cancer therapy and several new antibiotics. In the course of a bioactivity screening program of secondary metabolites produced by *Sorangium cellulosum* strains, the macrolide chlorotonil A was found to exhibit promising antimalarial activity. Subsequently, we evaluated chlorotonil A against *Plasmodium falciparum* laboratory strains and clinical isolates from Gabon. Chlorotonil A was highly active with a 50% inhibitory concentration between 4 and 32 nM; additionally no correlation between the activities of chlorotonil A and artesunate (Rho: 0.208) or chloroquine (Rho: -0.046) was observed. *Per oral* treatment of *P. berghei* infected mice with doses as little as 36 mg/kg chlorotonil A led to suppression of parasitemia with no obvious signs of toxicity. Chlorotonil A acts against all stages of intraerythrocytic parasite development, including ring stage parasites and stage IV-V gametocytes and requires only very short exposure to the parasite to exert its antimalarial action.

Conclusively, chlorotonil A has an exceptional and unprecedented profile of action and represents an urgently required novel antimalarial chemical scaffold. Therefore, we propose it as a lead structure for further development as an antimalarial chemotherapeutic.

Introduction

Malaria is the most important parasitic disease worldwide with an estimated 207 million cases causing 627 000 deaths in 2012 (1). Among *Plasmodium spp.* causing malaria in humans, *Plasmodium falciparum* is responsible for almost all severe and fatal cases and is the predominant species in Sub-Saharan Africa. Even though scaling up of malaria control programs led to a reduction in incidence and mortality rates (1) and first generation vaccines show some efficacy (2, 3), chemotherapy remains the mainstay of treatment for all forms of malaria. A major threat for chemotherapy is the development of resistance as it has been described for most registered antimalarials resistant phenotypes (4). In Sub-Saharan Africa, resistance against the former first-line drugs chloroquine and sulfadoxine-pyrimethamine is widespread (5) and decreased activity of artemisinin derivatives is well documented in South East Asia (6). The loss of artemisinin activity is of particular concern since all current efforts to control malaria are based on this class of compounds (7). To keep pace with the parasite's ability to develop resistance, a continuous effort to develop new drugs is crucial, especially in case of severe malaria for which only two alternative treatments (artesunate and quinine) are available (8).

Treatment and pharmacological requirements of drugs to treat uncomplicated or severe malaria differ fundamentally. Drugs for uncomplicated malaria should be available as an oral formulation, be easily administered (ideally once), and have a very good safety profile (e.g. wide therapeutic range, low toxicity especially in children and pregnant or lactating women, and few unwanted side effects); ideally they protect the individual for a prolonged time-period and protract resistance development. In contrast, patients with severe and complicated malaria need fast acting and usually parenterally administered drugs, which rapidly reduce parasite burden and display activity against all parasite isolates within a narrow concentration range. Their pharmacokinetic profile should allow parenteral administration in critically ill patients with organ failures. Since children are the most affected group, all antimalarials must be safe in this age group and be available in pediatric formulations (9).

To control malaria on the epidemiological level and prevent the spread of resistant parasites, antimalarial drugs should act on gametocytes, the sexual stages of the parasite to block transmission to the mosquito. However, most current antimalarials do not act against gametocytes, and transmission of the parasite is not prevented (10). Nevertheless, gametocytocidal activity is highly desirable for new antimalarials that shall be used in elimination and eradication programs (11).

In the past, characterization and development of natural compounds and their derivatisation was a particularly powerful way to find new chemotherapeutics against infectious diseases (12). One reason for the success rate with natural products may be the long-lasting interaction of co-evolving organisms resulting in structural classes optimized for their respective targets. In malaria, most highly-active compounds are plant-derived and are thought to have a role in deterring herbivores (e.g. quinine) or are potent herbicides (e.g. artemisinin) (13-16). Besides plants and fungi, bacteria including the soil-dwelling myxobacteria (17, 18) are a rich source of biologically active compounds (19-23). We investigated the antimalarial properties of a chlorine-containing metabolite, chlorotonil A, whose antiplasmodial activity was primarily identified when screening a library of myxobacterial substances at the Swiss Tropical and Public Health Institute. Chlorotonil A is a tricyclic macrolide produced by *Sorangium cellulosum* and was first isolated and described by Gerth et al. (24). Total synthesis of the substance and its dehalogenated derivative was reported recently (Figure 1) (25). In our study we found chlorotonil A to exhibit potent *in vitro* and *in vivo* activity against *P. falciparum* and *P. berghei*, respectively. It acted against all erythrocytic stages of the parasite, showed very rapid onset of action and was active *in vitro* against stages responsible for transmission.

Material and Methods

Parasite culture

P. falciparum strains 3D7 (chloroquine-sensitive) and Dd2 (chloroquine, sulfadoxine and

pyrimethamine resistant) were maintained in continuous culture as previously described (26). In brief, 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 gentamycin, and 0.5% w/v albumax) at 37°C, 5% CO_2 and 5% oxygen at 5% hematocrit with daily change of medium. Synchronization was performed by sorbitol twice a week (27).

Chemicals

Chlorotonil A (MW: 479.44) was isolated as described before (24), a compound without chlorine-atoms (MW: 410.54) was obtained by dehalogenation (see structures in Figure 1). Stock solutions of chlorotonil A and its dehalogenated form were prepared at 2.1 mM and 2.4 mM in tetrahydrofuran (THF). The comparator drug artesunate (Shin Poong, MW: 384.4) was prepared in 70% ethanol at 15 mM; chloroquine diphosphate (Sigma, MW: 515.86) in double-distilled water at 9.7 mM; epoxomicin (Sigma, MW: 554.7) and dihydroartemisinin (Shin Poong, MW: 284.35) in dimethylsulfoxide (DMSO) at 1 mM and 20 mM, respectively. Further dilutions of all drugs were done in complete culture medium. All solvent dilutions used did not interfere with parasite growth in pilot experiments. All results given are in nM.

***In vitro* drug sensitivity assay of laboratory strains**

Drug sensitivity assays were performed according to standard procedures (28). In brief, 96 well plates were pre-coated with a twofold serial dilution of the respective drug. Ring-stage parasites were diluted to a parasitemia of 0.05% with 0+ erythrocytes and seeded at a hematocrit of 1.5% in a total volume of 225 µl per well. After 3 days plates were freeze thawed twice and analyzed by measurement of histidine rich protein 2 (HRP2) with an enzyme-linked immunosorbent assay (ELISA). To measure delayed activity of the drug, parasites were incubated for 6 days (29); on day 2 and 4 medium was changed (140 µl) without replacement of drug. All experiments were done in duplicate and repeated at least three times.

***In vitro* drug sensitivity assay of clinical isolates**

We tested the activity of chlorotonil A together with standard drugs (artesunate, chloroquine) against *P. falciparum* clinical isolates from patients with uncomplicated malaria in Lambaréné, Gabon between February and May 2009. The investigations of the *in vitro* drug sensitivity of clinical isolates were approved by the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné. Assent and informed consent were obtained from the child and their legal representative, respectively. A venous blood sample was taken in a lithium heparin tube and processed within 4 hours. Assays were performed as in lab strains with minor modifications. Plates were pre-coated with a threefold dilution of each drug and parasites were incubated in a candle jar for 3 days at 37°C. Assays were performed only once, directly after blood draw. Only samples in which the amount of detected HRP2 at least doubled within the 3 days were included in the analysis.

Stage specific analysis

Synchronized parasites at the ring, trophozoite and schizont stage were incubated with the respective drug at 1.5% hematocrit and a parasitemia between 1 – 4% in a 96 well plate for a total of 40 hours. Samples were taken every 8 hours to determine parasite development microscopically by Giemsa (Sigma) stained thin blood smear. Artesunate (20 nM) was used as a positive control for stage specific action against rings and trophozoites. For action against the schizont stage, epoxomicin (500 nM) and artesunate (500 nM) were used as control. Chlorotonil A was used at a concentration of 40 nM for determination of action against ring and trophozoite stages and in addition at 500 nM against schizont stages.

Evaluation of onset of action

Assays were performed as for the *in vitro* standard drug sensitivity assay with laboratory strains but with removal of the drug after one hour. In detail: Synchronized ring stage 3D7 parasites were seeded at 0.05% parasitemia and 1.5% hematocriton pre-coated 96-well plates (chlorotonil A, artesunate and chloroquine in a threefold serial dilution). After one hour of incubation drugs

were removed by 3 times washing with complete medium. A control plate omitting the washing step was incubated in parallel to compare results. Subsequently incubation of plates was continued for 3 days as for the standard assay before the HRP2-ELISA was performed. The 50% inhibition concentrations of the one hour pulsed and standard plates of 3 independent experiments were compared. Differences in IC_{50} values are presented as mean fold change increase ± SD in respect to the control plates. All experiments were performed in duplicate.

In vivo efficacy

In vivo antimalarial activity of chlorotonil A was assessed against the rodent malaria strain *P. berghei* ANKA (provided by David Walliker, University of Edinburgh, UK) in BALB/c and Swiss CD1 mice in the four day suppression test (30, 31). In brief: 4 BALB/c mice and 5 Swiss CD1 mice were inoculated intravenously with $2x10^7$ parasitized erythrocytes (diluted in phosphate buffer saline (PBS)) obtained from a donor mouse. After infection, mice were treated at 2 h, 24 h, 48 h and 72 h post-infection with different amounts of powder of chlorotonil A (BALB/c: 36, 68, 110 mg/kg, one control; Swiss CD1: 3 mice received 100 mg/kg, two controls) in 100 mg of peanut butter (Barney`s Best) or placebo control (peanut butter only). Peanut butter with or without chlorotonil A was given each mouse individually out of a syringe. Thin blood smears from tail blood were taken daily from day 1 to day 5 and stained with 20% Giemsa (Sigma). A minimum of 1000 erythrocytes per slide was counted microscopically to assess parasitemia. The activity is expressed as percent reduction of parasitemia in comparison to the control group according to the following equation:

$$\text{Activity} = 100 - \frac{mean\ parasitemia\ treated}{mean\ parasitemia\ control} \times 100$$

Activity is analyzed on day 4; additionally we analyzed activity on day 5 which is recommended for slow acting drugs (31). Mice experiments were approved by the competent authority for animal experiments in Tübingen (no. T1/08) and performed according to the German legislation.

Gametocytocidal activity

Gametocytocidal activity of chlorotonil A was evaluated by an ATP bioluminescence assay as described previously (32). As comparators artesunate, epoxomicin and dihydroartemisinin were tested. In brief: gametocyte culture was initiated from synchronized 3D7 parasites with an increased concentration (0.75% w/v) of AlbuMax II solution, starting with 10% hematocrit and 0.5% parasitemia. Culture medium was changed daily without parasite dilution for the entire process. When the parasitemia reached 5%, the volume of the medium was doubled and concentration of AlbuMax II reduced to 0.5% w/v. Between day 11 and 14, cultures were treated with 50 mM N-acetyl-D-Glucosamine (MP Biomedicals GmbH) to remove asexual stages and on day 15 the culture was purified by NycoPrep 1.077 cushion density gradient and magnetic column separation in order to remove erythrocytes and enrich the gametocyte population. Compounds were pre-coated in a threefold dilution in 96 well plates before 50 000 gametocytes were added to each well in a final volume of 100 µL and incubated at 37°C, 5% CO_2 and 5% oxygen. After 48 hours, ATP production of the gametocytes was measured by the BacTiterGlo assay (Promega) according to the manufacturer's protocol and recorded by VICTOR^3 V Multilabel Reader (PerkinElmer, Inc.). All experiments were repeated at least three times.

Statistics

Individual inhibitory concentrations were determined by non-linear regression analysis of log-concentration-response curves using the drc-package v0.9.0 (33) of R v2.3.1 (34). Mean 50% and 90% inhibition concentration and standard deviations are presented for each drug assayed in laboratory strains. In clinical isolates the median 50% and 90% inhibition concentration and the range is given. Correlations between IC_{50}s of clinical isolates of the three different drugs were calculated with Spearman's (nonparametric) test for paired samples in JMP v5.0.1.2 software.

Results

Chlorotonil A acts against laboratory and clinical *P. falciparum* isolates *in vitro*

Chlorotonil A potently inhibits growth of chloroquine sensitive (3D7) and resistant (Dd2) *P. falciparum* parasite strains *in vitro* (Table 1). As reference compounds, chloroquine and artesunate were analyzed. To assess if chlorotonil A shows signs of hysteresis (also called delayed death phenomenon) similar to some other antimalarial antibiotics, a six-day-assay covering two intraerythrocytic cycles of parasite replication was performed. The IC_{50} obtained in the six day assay was 10.6 ± 4.1 nM (3D7) and 23.5 ± 9.4 nM (Dd2), the IC_{90} was 13.8 ± 6.4 nM (3D7) and 32.4 ± 13 nM (Dd2), indicating that chlorotonil A acts directly upon first contact with the parasite. The dehalogenated form of chlorotonil A showed no measurable activity against Dd2 and 3D7, even at the highest concentration tested (540 nM).

To assess variability of the activity in clinical isolates of *P. falciparum*, we measured activity of chlorotonil A against parasites freshly isolated from patients with uncomplicated malaria in Lambaréné, Gabon. Of 28 collected clinical isolates, 25 (chlorotonil A; chloroquine) and 26 (artesunate) were successfully grown in culture and could be analyzed in the *in vitro* assay. Chlorotonil A was active against clinical isolates with IC_{50}s comparable to those obtained against laboratory strains; in addition the determined IC_{50} values for the clinical isolates showed a narrow range (Table 2). No correlation between the activities of chlorotonil A and artesunate (Rho: 0.208, N =25) or chloroquine (Rho: -0.046, N =25) were observed.

Chlorotonil A acts against all blood stages of the parasite

To investigate effects of chlorotonil A on the morphology of different stages we incubated synchronous 3D7 ring, trophozoite and schizont stages with the drug and stained thin blood smears by Giemsa stain after 8, 24 and 40 hours. Chlorotonil A arrested parasite development of ring and trophozoite stages. Morphologically, parasites looked similar to artesunate-treated parasites since their development stopped immediately after contact with the compound (Figure 2A and Figure 2B). When added to schizont stage parasites, higher concentrations (>40 nM)

were required. Incubation with 500 nM chlorotonil A led to an arrest in development, which is comparable to proteasome inhibitors that act against the schizont stage, e.g. epoxomicin (Figure 2C). Notably, artesunate added at the same concentration (500 nM) did not block the egress of parasites and furthermore, erythrocytes newly infected by merozoites, were found (notice the presence of ring stage parasites after 8 hours in Figure 2C). The chlorotonil A-induced arrest in the schizont stage was apparent after 8 hours by thin blood smear. At later time points arrested schizonts showed signs of degradation.

Chlorotonil A acts fast

The standard *in vitro* susceptibility assay does not account for short drug-pulses but *in vivo* drug concentration fluctuates and high concentrations are maintained for limited time periods only. Therefore we incubated ring stage parasites with chlorotonil A or the comparator drugs artesunate and chloroquine for one hour, removed the drug and continued incubation as in the standard drug sensitivity assay. The IC_{50} of a one-hour pulse of chlorotonil A was only 1.3 (± 0.7) fold higher when compared to the standard assay. In contrast, the IC_{50} of a one-hour pulse of artesunate was 15.6 (± 9.1) fold higher compared to the standard assay. As expected, chloroquine was not active when given as a short pulse at ring stage, even at the highest concentration tested (160 µM).

Chlorotonil A is active *in vivo*

Chlorotonil A is not soluble in most commonly used solvents. Nevertheless, a pilot experiment to assess therapeutic efficacy of non-solubilized, orally administered chlorotonil A was performed in *P. berghei* ANKA infected BALB/c mice and Swiss CD1 mice using Peters 4 day suppression test (31). Activity of chlorotonil A on Day 4 was 97% in BALB/c mice and 98% in Swiss CD1 mice when compared to control mice. On day 5 anti-plasmodial activity was 93% (BALB/c) and 85% (Swiss CD1), respectively. However, cure was not complete with the standard protocol, as none of the mice cleared parasitemia completely (Figure 3). In BALB/c mice, all doses (36, 68, and 110 mg/kg) resulted in a substantial reduction of parasitemia. This reduction showed some dose-

dependency; however due to the small sample size this was not analyzed formally. Mice did not show obvious signs of toxicity due to chlorotonil A treatment at either dose.

Chlorotonil A is active against gametocytes

To assess the activity of chlorotonil A against stage IV-V gametocytes, we used an *in vitro* bioluminescence assay (32). Chlorotonil A was active against late stage gametocytes at similar concentrations as against asexual blood stages. This is in contrast to artesunate and dihydroartemisinin, which act only when used in very high concentrations (Table 3). Epoxomicin served as an internal control for the assay since it is known to be highly active against stage IV-V gametocytes.

Discussion

So far, continuous development of new drugs remains the only option to keep pace with the potential of *P. falciparum* to adapt to man-made interventions. Myxobacteria, especially the genus *Sorangium* has proven to be a valuable source for new chemotherapeutic compounds such as soraphens, sorangicins, thuggacins, and epothilones (35-37), some of which are in advanced preclinical and clinical development. For example ixabepilone (BMS-247550), an analogue of epothilone B, was approved by the Food and Drug Administration in 2007 for the treatment of breast cancer (38). *Sorangium cellulosum* strain So ce1525 was identified as our main producer of chlorotonil A and yields several natural products exhibiting different chemical scaffolds. New scaffolds are of particular interest for the development of new antimalarials, since most compounds in the development pipeline belong to a restricted number of drug classes. Chlorotonil A shows several remarkable features, which characterize it as promising lead compound. Apart from being active against chloroquine-sensitive and chloroquine-resistant strains at low nanomolar concentrations, it is acting against all blood stages of the malaria parasite and does not show a hysteresis effect, which is a characteristic of several antimalarial antibiotics (39, 40). Chlorotonil A acts against early ring and trophozoite stages of the parasite,

and is therefore able to reduce parasite biomass and the generation of parasite toxins immediately upon contact with the parasite (41). This is of note since it is one characteristic that needs to be fulfilled by drug candidates for the treatment of severe malaria; also artemisinin derivatives have similar profile of action. The mode of action of chlorotonil A is not known but the loss of activity observed for the dehalogenated derivative points towards an important role of the chlorine containing pharmacophore. It is also acting against clinical isolates with different genetic background, isolated from patients in Lambaréné, Gabon, an area of high-grade resistance against chloroquine and sulfadoxine –pyrimethamine (42, 43). The lack of correlation with its comparator drugs chloroquine and artesunate supports the assumption that chlorotonil A has a different mechanism of action than these two drugs.

First tests in the murine *P. berghei*-model demonstrated that chlorotonil A is active *in vivo*, has a low toxicity and can be administered orally. Due to its poor solubility, it was given to the mice as a powder together with peanut butter because tetrahydrofuran, the solvent used for *in vitro* assays, is toxic for mice (44). Currently, we explore alternative formulations and chemical modifications to develop chlorotonil A from an early lead towards a drug development candidate. Besides that it would be interesting to test anthracimycin, a structural relative of chlorotonil A and its dichloro derivative (45).

An exceptional property of chlorotonil A is its very rapid onset of action: one hour contact with the parasite is sufficient to exert its full activity. The two comparator-drugs, artesunate and chloroquine, had strongly reduced activity when pulsed for one hour only. The rapid onset as seen for chlorotonil A is especially important for drugs which are used to treat severe malaria, where a rapid reduction of parasitemia is vital for the survival of the patient. In addition rapid onset is important for drugs with a short half-life like artesunate and dihydroartemisinin. Both exhibit half-lives shorter than one hour (46) and thus, the timespan of therapeutic drug concentration above the antiparasitic threshold is short. This is one of the reasons why cure rates of artemisinin derivatives are poor when given for less than 5-7 days as monotherapy (47).

Activity against late stage gametocytes is an additional valuable feature of chlorotonil A. Late stage gametocytes are especially difficult to target by drugs. Until now, primaquine is the only licensed drug with known activity against them *in vivo*, but its hemolytic effect in individuals with glucose-6-phosphate dehydrogenase deficiency limits its use (48). Artemisinin derivatives decrease gametocyte carriage *in vivo* mainly by their rapid action accompanied by the reduction of parasite biomass but it is shown that they cannot stop transmission completely (49, 50). The effect of artemisinin derivatives on the gametocytes is still under discussion; they probably also act against early gametocytes but only little activity against late stage gametocytes is reported (32, 51-53). This is also in concordance with our assay. Especially when compared side by side with chlorotonil A, the activity is orders of magnitude lower. A new gametocytocidal drug would be a major step forward for the feasibility of elimination and resistance containment campaigns, especially in low endemicity settings where transmission blocking is crucial to finally stop the cycle.

Chlorotonil A exhibits the promising characteristics of a potential new lead compound with its low toxicity, oral availability, rapid onset of action and activity against all erythrocytic stages of the parasite. In addition it is active against the transmission stages of the parasite. Improved derivatives and dose regimens are required prior to clinical development but already at this stage it is evident that chlorotonil A has unique features that warrant its further assessment.

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Figures

Figure 1: Chemical structure of chlorotonil A and its dehalogenated form.

Figure 2: Stage specific inhibition of *P. falciparum* by chlorotonil A.

Giemsa stained thin blood smears of synchronous ring (Figure 2A), trophozoite (Figure 2B) or schizont (Figure 2C) *P. falciparum* 3D7 parasites after different incubation times (8, 24 and 40 hours) with chlorotonil A and artesunate (ring, trophozoite stage), and epoxomicin and artesunate (schizont stage) at the indicated concentrations compared to the drug free control (enlarge picture to better differentiate stages).

Figure 3: Parasitemia of *P. berghei* in chlorotonil A treated mice.

Parasitemia (%) in four *P. berghei* infected BALB/c (balbc/1 - balbc/4, indicated by dotted lines) and five Swiss CD1 (swiss/1 - swiss/5, indicated by solid lines) mice from day 1 to day 5 in the four day suppression test. Mice indicated by black lines received either 36, 68, 110 mg/kg (BALB/c) or 100 mg/kg (Swiss CD1) chlorotonil A and mice indicated by the red line received placebo control.

Table 1: Mean Inhibitory concentrations (IC_{50} and IC_{90} in nM) and standard deviations of tested compounds against the *P. falciparum* strains 3D7 and Dd2.

	3D7		**Dd2**	
	IC_{50}±SD	**IC_{90}±SD**	**IC_{50}±SD**	**IC_{90}±SD**
Chlorotonil A	9.1±3	13.34±3.2	18.1±8.6	28.5±8.5
Chloroquine	5.2±1.2	8.2±2.3	160.4±62.1	228.6±66.4
Artesunate	2.4±1.3	7.5±4.6	1.3±0.2	1.8±0.2

Each value is the mean IC of at least 3 independent experiments performed in duplicates.

Table 2: Median inhibitory concentrations (IC_{50} and IC_{90} in nM) and range of 25 (chlorotonil A, chloroquine) and 26 (artesunate) different clinical isolates against the respective compounds. Each experiment is performed once.

	IC_{50}(range)	**IC_{90}(range)**
Chlorotonil A	15.2 (3.7-32)	37.1 (6.8-76.2)
Chloroquine	47.2 (19.5-117)	111.6 (44.2-191.1)
Artesunate	0.6 (0.2-3.2)	1.8 (0.6-4.7)

Table 3: Mean inhibitory concentrations (IC_{50} and IC_{90} in nM) and standard deviations of tested compounds against stage IV-V gametocytes of *P. falciparum* strain 3D7.

	IC_{50}±SD	**IC_{90}±SD**
Chlorotonil A	29.6±16.3	123.2±36.9
Artesunate	8 917±4 830	25 852±19 563
Dihydroartemisinin	2 918±964	16 603±16 141
Epoxomicin	3.2±2.6	14.3±16.7

Each value is the mean IC of at least 3 independent experiments.