DNA Methylation Bisubstrate Inhibitors Are Fast-Acting Drugs Active against Artemisinin-Resistant Plasmodium falciparum Parasites

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Supporting Information

ABSTRACT: Malaria is the deadliest parasitic disease affecting over 200 million people worldwide. The increasing number of treatment failures due to multi-drug-resistant parasites in South-East Asia hinders the efforts for elimination. It is thus urgent to develop new antimalarials to contain these resistant parasites. Based on a previous report showing the presence of DNA methylation in Plasmodium, we generated new types of DNA methylation inhibitors against malaria parasites. The quinoline–quinazoline-based inhibitors kill parasites, including artemisinin-resistant field isolates adapted to culture, in the low nanomolar range. The compounds target all stages of the asexual cycle, including early rings, during a 6 h treatment period; they reduce DNA methylation in the parasite and show in vivo activity at 10 mg/kg. These potent inhibitors are a new starting point to develop fast-acting antimalarials that could be used in combination with artemisinins.

INTRODUCTION

Malaria is a global health problem, still causing 435,000 deaths each year and over 200 million cases according to WHO. Considering the lack of an efficient vaccine and the increasing threat of resistance in the parasite and vector, new drugs that target resistant parasites are of the highest priority. In the lethal human malaria parasite, Plasmodium falciparum, epigenetic gene regulation governs the stage-specific biology of the parasite as it develops within the human host and mosquito vector. This regulation is via multiple mechanisms: specialized nuclear architecture, histone modifications and variants, and chromatin-associated noncoding RNAs. Epigenetic changes confer to this protozoan pathogen a phenotypic plasticity that characterize its proliferation and progression throughout the different stages of its life cycle. Targeting histone modifications was shown to interfere with various life cycle stages, and we have previously identified small molecules able to block parasite growth and interfere with dormancy. The identification of DNA cytosine methylation and more recently hydroxymethylation in P. falciparum makes these modifications a potential drug target to develop new antimalarials. The genome of P. falciparum contains only one bioinformatically predicted gene with a DNA methyltransferase (DNMT) domain (PF3D7_0727300) that is related to the DNMT2 enzyme family. In 2013, Ponts et al. reported low-level DNA cytosine methylation activity of a recombinant PfDNMT2 domain, whereas Govindaraju et al. noted that the recombinant PfDNMT2 domain methylates tRNA. Thus, the biological function of PfDNMT2 in DNA methylation remains ambiguous and points to the existence of a noncanonical DNMT enzyme in malaria parasites. DNMT inhibitors could be excellent chemical tools to study the DNMT pathway in P. falciparum. Recently, quinazoline-based inhibitors of human DNMT3a showed antimalarial activity, but their plasmodial target is unknown. These quinazolines are derivatives of BIX-01294, a compound with potent antimalarial...
activity and a known inhibitor of human histone methyltransferase G9A that we have previously studied and characterized. We have previously identified quinoline-quinazoline derivatives, namely, bisubstrate analogues, as inhibitors of human DNMT3a (Figure 1a) and DNMT1. Bisubstrate inhibitors are based on the design of analogues of the substrate pocket and the cytidine pocket (in blue in Figure 1a), and of analogues of the cofactor pocket, S-adenosyl-L-methionine (in red), linked together through different linkers (in green), resulting in a potent inhibitor of the enzyme. As the DNMT catalytic pocket is highly conserved, we tested this novel family of inhibitors in parasite proliferation assays. Here, we show that these compounds are potent growth inhibitors of all P. falciparum asexual blood stages and reduce DNA methylation in Plasmodium. These compounds kill also multi-drug-resistant P. falciparum at the low nanomolar level, including artemisinin-resistant strains. One compound is active at 10 μg/kg in mice infected by Plasmodium berghei.

## RESULTS

### Identifying DNMT Bisubstrate Inhibitors as Potent Antimalarials

We generated an in-house chemical library of human DNMT inhibitors, and we screened 71 compounds against the proliferation of P. falciparum drug-sensitive strain NF54. Five hits were found belonging to the bisubstrate family (Figure 1, nomenclature as in Halby et al., 2017). We determined their IC\(_{50}\) (Table 1) and identified the most potent compounds 20 and 70 with an IC\(_{50}\) of 71 ± 23 and 60 ± 14 nM, respectively. These two compounds have a selectivity index of 20 and 42, respectively, when compared to the human HepG2 cell line.

### Bisubstrate Analogues Decrease DNA Methylation in P. falciparum

We adapted the highly sensitive in vitro DNA methylation assay, that we previously developed, to measure P. falciparum DNA methylation in nuclear extracts. The compounds were tested at a concentration of 32 μM that we typically use for screening. Compounds 20 and 70 significantly inhibit the capacity of the extracts to methylate DNA, whereas inactive compound 1 and dihydroartemisinin (DHA) were used as negative controls (Figure 1b), mimicking the results obtained on hDNMT3a-C. These observations show that compounds 20 and 70 are on target in P. falciparum. The residual methylation activity observed can be due to the competition of the plasmodial DNMT with other nonspecific DNA binding proteins and the presence of demethylase activity. In fact, we recently showed that TET demethylase activity is present in P. falciparum nuclear extracts.

### Activity against Cambodian Multi-Drug-Resistant Strains

We evaluated the ability of compounds 20 and 70 to inhibit the proliferation of multi-drug-resistant field isolates adapted to culture. A panel of four Cambodian strains (S150, 6591, S248, 6320) bearing different molecular markers of resistance were selected (see Table S1). All four are resistant to chloroquine and pyrimethamine, and two present the following additional resistance phenotypes: S248, mefloquine and artemisinin (Kelch13 C580Y); 6320, piperaquine and artemisinin (Kelch13 C580Y). The drug-sensitive laboratory strain 3D7, cultured in the same conditions (5% human serum), was adapted to culture. A panel of four Cambodian strains (5150, 6591, 5248, 6320) bearing dihydroartemisinin (DHA) were used as negative controls (Figure 1b), mimicking the results obtained on hDNMT3a-C. These observations show that compounds 20 and 70 are on target in P. falciparum. The residual methylation activity observed can be due to the competition of the plasmodial DNMT with other nonspecific DNA binding proteins and the presence of demethylase activity. In fact, we recently showed that TET demethylase activity is present in P. falciparum nuclear extracts.

### Effect on the Parasite Cell Cycle

To determine stage-specific targeting of the asexual cycle, synchronized parasites were treated in 8 consecutive 6 h windows, spanning the 48 h
asexual cycle, with different concentrations of 20 and 70 (Figure 2c). The compounds are active throughout the 48 h cycle with an increased potency between 12 and 36 h postinvasion (hpi). Compound 70 was more potent. Even at the IC50 concentration the inhibitors kill the parasite in 6 h, even in the early ring stage where compounds 20 and 70 kill up to 50% and 90%, respectively. These compounds thus show a fast-clearing profile against all stages, comparable to what is described with artemisinin derivatives.

Activity in the Ring-Stage Survival Assay. Since the compounds are active on the early ring stage (0−6 hpi parasites), we tested their ability to kill the artemisinin-resistant strains 5248 and 6320 in the ring-stage survival assay (RSA) (Figure 2d). Both compounds kill resistant rings at 3 times the 3D7-derived IC50 value in strain 5248, comparable to their effect in the sensitive cell line NF54 (Figure 2c, 0−6 h treatment window). Both compounds, and in particular compound 20, were less efficient in strain 6320 that is resistant to artemisinin and piperaquine. Nevertheless, the compounds efficiently kill all the parasites, even in this resistant strain, when used in combination with 700 nM DHA.

Activity in Combination with Dihydroartemisinin. Since combining compounds 20 and 70 with DHA enables the efficient killing of the resistant parasites in the ring-stage survival assay, we wanted to characterize the effect of this combination in the 3D7 and piperaquine-resistant 6320 strains...
using the fixed-ratio isobologram method. In both strains, we obtained isobolograms close to the line 1:1 for compound 20 and 70 (Figure 2e), indicating an additive effect.

**In Vivo Activity.** To study the effect on malaria parasites in vivo, we examined parasite clearing in an acute infection murine model (P. berghei ANKA strain parasites). ANKA strain parasites are highly virulent, normally causing death by cerebral malaria within 1 week after injection. Compound 20 was chosen because it showed better solubility in water at the higher concentrations required in vivo. On day 1, 2, and 3 postinfection, mice were injected i.p. with a dose of 20 (10 mg/kg), or chloroquine (25 mg/kg), or the vehicle control. Parasitemia was followed daily by flow cytometry. Compound 20 is able to clear the infection for the 6 days measured. One mouse in the test group died at day 6, perhaps due to toxic effects of the treatment whereas all mice succumbed to cerebral malaria in the control group (Figure 2e).

**DISCUSSION**

Here, we show that DNA methylation inhibitors have a strong efficacy against the human malaria parasite *P. falciparum*. Our findings demonstrate that targeting DNA cytosine methylation is a potent strategy to fight malaria. We show for the first time that quinoline–quinazoline derivatives inhibit the capacity of *P. falciparum* nuclear extracts to methylate DNA, showing that these compound target DNA-methylation in *Plasmodium* (Figure 1b). The inhibition of DNA methylation could explain the pharmacological advantages observed, notably that bisubstrate inhibitors target all stages of the asexual blood cycle in 6 h (Figure 2c), making them attractive compounds with pharmacological properties close to artemisinins.

Most promising, the compounds are active in artemisinin-resistant Cambodian strains (Figure 2a,b,d). However, we observe some cross-resistance with piperazine, perhaps due to the similarity of the quinoline–quinazoline scaffold with that of piperazine (which consists of two 4-aminouquinolines attached by a linker). In future experiments, it would be interesting to study the mechanisms of this cross-resistance, such as the involvement of increased copy numbers of Plasmepsin II or PiCRT mutations (H97Y, C101F, F145I, M343L, G353V) found in South-East Asian isolates. Importantly, whereas piperazine is known to target the trophozoite stage and is not active on the ring stages in 6 h (data not shown), compound 70 rapidly kills the parasite in the early ring stages (Figure 2c). In addition, the association with DHA allows this cross-resistance to be overcome, suggesting that the parasites resistant to piperazine are distinct from the dormant parasites resistant to artemisinin. This association appears to be additive in the 3D7 strain and in the piperazine-resistant isolate (Figure 2e), suggesting a different mode of action between bisubstrate inhibitors and artemisinin. Considering that the first-line treatment, artemisinin-based combination therapies (ACTs), is failing due to resistances, having a combination that is able to efficiently kill the parasites resistant to the current ACTs is extremely valuable. Finally, compound 20 efficiently protects mice from mortality due to *P. berghei* (Figure 2f).

In conclusion, we show that bisubstrate inhibitors of DNA methylation have potent and fast antimalarial activity comparable to artemisinins and maintain their activity in artemisinin-resistant strains. We confirm that the compounds inhibit the DNA methylation activity of *P. falciparum*, identifying DNA methylation as a potent strategy to fight malaria in all blood stages, including the early ring-stage linked to artemisinin resistance. These potent inhibitors constitute a new starting point in the development of fast-acting antimalarials.

**MATERIALS AND METHODS**

The chemical synthesis of bisubstrate analogues was described in Halby et al., 2017. Other chemicals were purchased from Sigma-Aldrich.

**Cytotoxicity in the HepG2 Cell Line.** Human HepG2 hepatocellular carcinoma cells by DSMZ were grown as advised by the provider, and cytotoxicity was evaluated after 72 h of treatment in 96-well plates by ATPlite (PerkinElmer) according to the manufacturer’s protocol. The experiment was run in triplicate.

**P. falciparum Continuous Culture.** *P. falciparum* parasites were cultured using a standard protocol. The strains used were NF54 and 3D7, and a panel of four Cambodian isolates adapted to culture (S150, 6591, S248, 6320; see Table S1). These isolates have been collected in the framework of the therapeutic efficacy surveillance program in Cambodia from 2011 to 2013. PSA, RSA, and genotyping data have been performed in the Pasteur Institute in Cambodia. Genomic data and phenotypes of isolate 6320 have been published previously.

**Inhibition Activity in the Asexual Stages.** IC50 values were obtained as previously described. A range of 7-point and 2-step serial dilutions starting at 500 nM were used to assess the activity of compounds 20 and 70. GraphPad Prism 8 was used to interpolate IC50 from three independent experiments ran in triplicate. DHA and DMSO were used as positive and negative controls, respectively. Figure 2b was obtained by dividing the IC50 obtained in the Cambodian isolate by the one obtained in the 3D7 strain, in each independent experiment. Mean and SD are representative of three data obtained for each test and control compounds. The statistical analysis for compounds 20 and 70 was performed using the one sample test in comparison to the value 1 in GraphPad Prism 8.

**Stage-Specificity during the Asexual Cell Cycle.** To assess stage specificity, asexual NF54 parasites were tightly synchronized (0–3 hpi) using gelatin flotation (to purify late stages) followed by a 5% d-sorbitol treatment (to eliminate non-ring stages) 3 h later. The parasites were then dispersed in 48-well plates (500 μL per well at 2% hematocrit, 0.5% starting parasitemia) and incubated for 6 h with the compounds at 1X, 3X, and 10X IC50 value, either directly (0–6 h treatment window) or after 6, 12, 18, 24, 30, 36, or 42 hpi. Following each 6 h treatment, cells were pelleted, washed with 10 mL of RPMI, and put back into culture in 500 μL of complete media in a new plate. Parasitemia was assessed at 72 h post-synchronization using Giemsa-stained thin blood smears. The percentage of survival was compared to DMSO-treated parasites. Data were obtained from three independent experiments (one well per condition).

**Ring-Stage Survival Assay (RSA).** The RSA was determined as previously described. Briefly, 0–3 h synchronized ring-stage parasites were exposed to a 6 h treatment with either DMSO, 700 nM DHA, 3X IC50 of the inhibitors, or the combination of both. The IC50 used was the one obtained against the 3D7 strain. Parasitemia was assessed at 72 h post synchronization using Giemsa-stained thin blood smears. The percentage of survival was compared to DMSO-treated parasites, and data were obtained from three independent experiments (one well per condition).

**Fixed-Ratio Isobolograms.** The effect of the combination of compounds 20 and 70 with DHA was assessed following the
method described by Fivelman et al. 1, 13 Briefly, six solutions were prepared containing the following combinations: 0:0, 40:100, 30:200, 20:300, 10:200, and 0:500, respectively (concentration ratios of DHA to 20/70 in nanomolar, with the first and last solutions being each drug alone). These solutions were 2-step serially diluted in a range of 7-point, the last row being left for 0.1% DMSO-treated controls (row H). Asynchronous parasite culture containing mostly rings was added at 0.5% parasitemia and 2% final hematocrit. IC50 values were determined after 72 h of incubation. Fractional IC50 (FIC) values were calculated by dividing the IC50 obtained in the combination by the IC50 obtained with the compound alone. The FIC of each combination was then plotted to obtain the isobologram represented in Figure 2e. The data were obtained in two independent experiments ran in triplicate.

Preparation of P. falciparum Nuclear Extracts. P. falciparum nuclear extracts were prepared from 107 saponin-lysed infected RBCs, following the protocol described in Methods In Malaria Research (sixth edition), 14 with slight modifications. Briefly, a parasite pellet was resuspended in 1 mL of cytoplasmic lysis buffer (25 mM Tris-HCl pH 7.5, 10 mM NaCl, 1% igepal, 1 mM DTT, 1.5 mM MgCl2, and protease inhibitor cocktail) and incubated at 4 °C on rotation. Parasites were then transferred to prechilled douncer homogenizer and lysed with about 200 strokes. Nuclei were sedimneted by centrifugation at 16 000 g for 20 min at 4 °C, and supernatant containing the cytoplasmic fraction was aliquoted and snap frozen. For nuclear extraction, nuclei were resuspended in 100 μL of nuclear lysis buffer (25 mM Tris-HCl pH 7.5, 600 mM NaCl, 1% igepal, 1 mM DTT, 1.5 mM MgCl2, and protease inhibitor cocktail) and shaken vigorously for 30 min at 4 °C. Finally, 300 μL of cytoplasmic lysis buffer was added to a dilute salt concentration, and cell debris were pelleted by centrifugation at 20 000 g for 20 min at 4 °C. Supernatant containing the nuclear soluble proteins was aliquoted and snap frozen. Protein concentration was measured using a Bradford colorimetric protein quantification assay.

In Vitro hDNMT3a-C Enzymatic Assay and Activity of P. falciparum Nuclear Extracts. A DNMT enzymatic assay was performed following the method described in Gros et al., 2013, 13 and developed in Ceccaldi et al., 2011. 12 Briefly, DNMT inhibition by the compounds was evaluated by mixing 0.05 μM 40-mer FAM-biotin DNA (FAM-5′-GCTATATATACGT-CTGTGACCCTACGACATGCA-CTG-3′/BIOT-5′-CAGTGCATGTCTGGTAGG-TTCACAGTACGTAT-3′, Eurogentec) with 0.1 mg/mL P. falciparum nuclear extract and 20 μM cofactor S-adenosyl-L-methionine (SAM) (NEB B9003S) in the presence of test and control compounds (final concentration of 32 μM) or DMSO (0.1% final concentration). After incubation at 37 °C for 1 h and washing with PBST 0.5 M NaCl and PBST, an unmethylated DNA specific restriction was induced by SU of HpyCH4IV (NEB R0619L) for 1 h at 37 °C. After washing, the fluorescence was evaluated on an EnVision Multilabel reader (PerkinElmer). A 200 ng portion of recombinant human DNMT3a-C was used as a positive control. Data were run in triplicate in two independent experiments. Data are analyzed with GraphPad Prism 8.

In Vivo Activity. In vivo activity was determined as previously described 1 following the Peters 4 day suppressive test, 2, 26 with slight modifications. Five CL57BL/6 mice per treatment group were infected intraperitoneally (i.p.) with 106 P. berghei ANKA strain GFP-expressing parasites. 26 Then, 2 h postinfection, mice were treated with a daily regimen of 10 mg/kg i.p. injection of compound 20, or 25 mg/kg chloroquine, or the equivalent vehicle control (distillated water) for 3 days. Parasitemia was quantified from blood samples collected every day by flow cytometry of 50 000 RBCs and confirmed by Giemsa-stained blood smears.

Statistics. Statistical analysis was realized using GraphPad Prism 8. The unpaired t test (two-tailed) assuming unequal variances was used to determine statistical difference between compounds 1, 20, and 70 compared to the DMSO control in Figures 1b and 2c. Statistical analysis for Figure 2b was performed using the one sample t test in comparison to the value 1.

Safety Statement. No unexpected or unusually high safety hazards were encountered.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.9b00874.

Characteristics of the Cambodian multi-drug-resistant isolates adapted to culture (PDF)

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Notes

The authors declare no competing financial interest.

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