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

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

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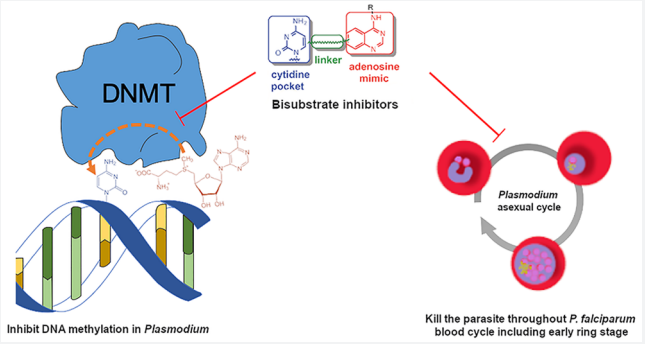
Supporting Information

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.[1] 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.[2] 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.[3,4] 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.[5,6] The identification of DNA cytosine methylation[7] and more recently hydroxymethylation[8] 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 *Pf*DNMT2 domain,[7] whereas Govindaraju et al. noted that the recombinant *Pf*DNMT2 domain methylates tRNA.[9] Thus, the biological function of *Pf*DNMT2 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.[10] These quinazolines are derivatives of BIX-01294, a compound with potent antimalarial

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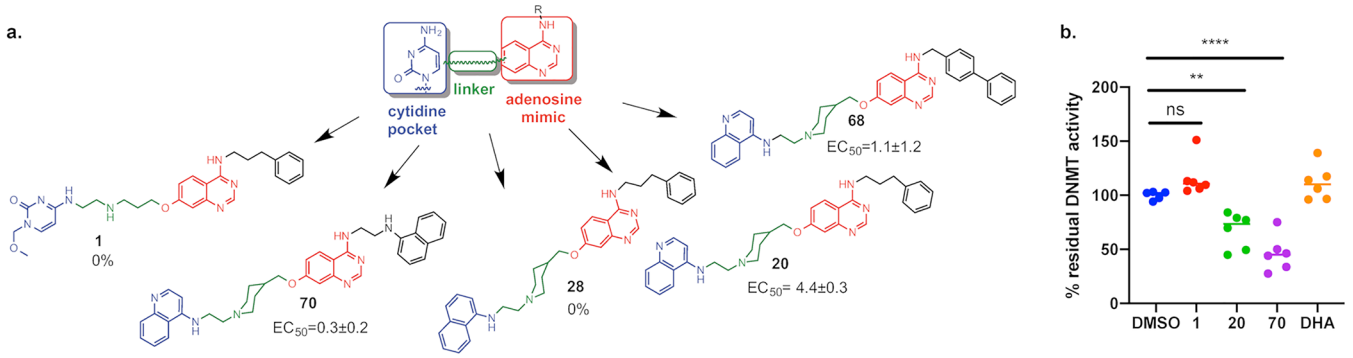

Figure 1. DNA-methyl transferase inhibiting activity of bisubstrate analogues. (a) Design and activity of quinazoline derivatives against human catalytic DNMT3a, hDNMT3a-C (EC_{50}, μM, data from Halby et al., 2017).[11] The compounds were designed as bisubstrate analogues to mimic the two DNMT substrates: *S*-adenosyl-L-methionine (in red) and deoxycytidine (in blue) linked by a linker (in green). The compounds are numbered according to Halby et al., 2017.[11] Compounds **1** and **28** have no activity at 32 μM (0% inhibition). (b) Inhibition of *P. falciparum* nuclear extract-mediated DNA methylation by compounds **1**, **20**, **70**, and DHA at 32 μM. Dots represent the value obtained in two independent experiments ran in triplicate. Statistical significance was determined using the unpaired *t* test in comparison to the DMSO control: ns, not significant; **, $p < 0.01$; ****, $p < 0.0001$. Statistical values: **1** ($p = 0.0766$, $t = 2.000$, df = 9); **20** ($p = 0.0020$, $t = 4.298$, df = 9); **70** ($p < 0.0001$, $t = 7.084$, df = 9).

activity and a known inhibitor of human histone methyltransferase G9A that we have previously studied and characterized.[5] We have previously identified quinoline−quinazoline derivatives, namely, bisubstrate analogues, as inhibitors of human DNMT3a (Figure 1a) and DNMT1.[11] 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 mg/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).[11] 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.

Table 1. Inhibition of the Proliferation of Asynchronous Asexual Cultures of *P. falciparum* NF54 and HepG2 Cells[a]

compound	*P. falciparum* (nM)	HepG2 cells (nM)
1	2376 ± 324	nd
20	71 ± 23	1400 ± 200
28	392 ± 13	nd
68	513 ± 63	nd
70	60 ± 14	2500 ± 500
dihydroartemisinin (DHA)	5 ± 1	nd

[a]Mean IC_{50} ± SD (standard deviation) of at least two independent experiments ran in triplicate. nd: not determined.

Bisubstrate Analogues Decrease DNA Methylation in *P. falciparum*. We adapted the highly sensitive *in vitro* DNA methylation assay, that we previously developed,[12,13] 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.[12,14] 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.[11] 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.[8]

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 (5150, 6591, 5248, 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: 5248, 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 used as a comparison. Figure 2a,b represents, respectively, the IC_{50} curves obtained in these strains for compounds **20** and **70** and the fold increase of IC_{50} obtained as compared to the 3D7 strain. The compounds were equally active against the four resistant strains and 3D7 (Figure 2a,b). The piperaquine-resistant strain 6320 was slightly less sensitive (orange curve in panel a), raising the possibility that piperaquine resistance decreases the activity of the bisubstrate analogues. The cross-resistance can be overcome since compound **70** remains very active reaching 0% growth of this strain at 500 nM (orange curve on the right).

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

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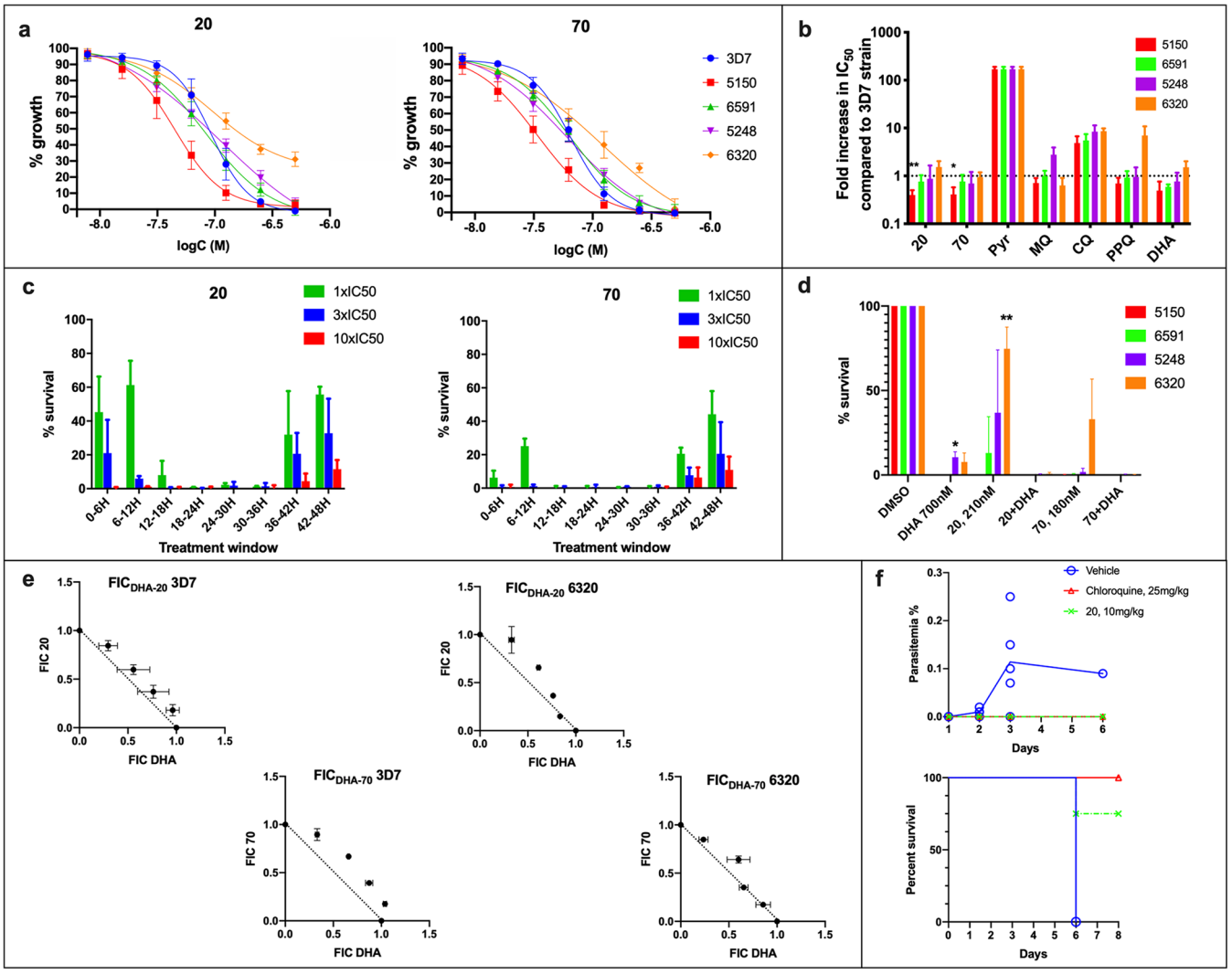

Figure 2. Antiplasmodial activity. (a) Growth inhibition curves of compounds **20** and **70** against multi-drug-resistant *P. falciparum* Cambodian isolates adapted to culture and the laboratory strain 3D7. (b) Fold increase in IC_{50} against multi-drug-resistant strains compared to 3D7. The dotted line intersects the value 1 that represents no effect. Statistical deviation from the value 1 essayed for compounds **20** and **70** is significant only for the 5150 strain ($p = 0.0097$ and $p = 0.0252$, respectively) where an increased sensitivity is observed (one sample *t* test). (c) Stage-specific activity in *P. falciparum* NF54 asexual cell cycle. 0−3 h synchronized parasites were incubated with the drug for 6 h, every 6 h of the 48 h cell cycle, using three different concentrations (1×, 3×, and 10× IC_{50}). Parasitemia was read at 72 h. (d) Ring-stage survival assay of **20** and **70** alone (3× IC_{50}) or in combination with 700 nM DHA (140× IC_{50}). Statistical significance in comparison to the value obtained in strain 5150 is marked with asterisks (* = 0.0285; ** = 0.0097; unpaired *t* test assuming nonequal variances). (e) Isobolograms obtained for the combination of compound **20** (top) or **70** (bottom) and DHA in the strains 3D7 (drug-sensitive) and 6320 (piperaquine-resistant). The dotted line represents the intersect of the fractional IC_{50} (FIC) 1:1, representing an additive effect. (f) *In vivo* activity of compound **20** in C57BL/6 mice infected with *P. berghei* ANKA. Data represented in this figure are the mean and standard deviation of three independent experiments (three technical replicates for panels a and b; no technical replicates for panels c and d); panel e is the mean and standard deviation of two independent experiments ran in triplicate, and *in vivo* data represent one experiment representative of the results obtained in two independent experiments (5 mice per group).

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 IC_{50} 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 IC_{50} 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

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using the fixed-ratio isobologram method.[15] 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[11] 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 piperaquine, perhaps due to the similarity of the quinoline−quinazoline scaffold with that of piperaquine (which consists of two 4-aminoquinolines 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 PfCRT mutations (H97Y, C101F, F145I, M343L, G353V) found in South-East Asian isolates.[16−20] Importantly, whereas piperaquine is known to target the trophozoite stage[21] 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 enables this cross-resistance to be overcome, suggesting that the parasites resistant to piperaquine are distinct from the dormant parasites resistant to artemisinin. This association appears to be additive in the 3D7 strain and in the piperaquine-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.[11] 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.[22] The strains used were NF54 and 3D7, and a panel of four Cambodian isolates adapted to culture (5150, 6591, 5248, 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.[16]

Inhibition Activity in the Asexual Stages. IC_{50} values were obtained as previously described.[5] 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 IC_{50} 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 IC_{50} 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 *t* 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 dispensed in 48-well plates (500 μL per well at 2% hematocrit, 0.5% starting parasitemia) and incubated for 6 h with the compounds at 1×, 3×, and 10× IC_{50} 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 postsynchronization 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.[23] Briefly, 0−3 h synchronized ring-stage parasites were exposed to a 6 h treatment with either DMSO, 700 nM DHA, 3× IC_{50} of the inhibitors, or the combination of both. The IC_{50} used was the one obtained against the 3D7 strain. Parasitemia was assessed at 72 h postsynchronization 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

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method described by Fivelman et al.[15] Briefly, six solutions were prepared containing the following combinations: 50:0, 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. IC_{50} values were determined after 72 h of incubation. Fractional IC_{50} (FIC) values were calculated by dividing the IC_{50} obtained in the combination by the IC_{50} 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 10^9 saponin-lysed infected RBCs, following the protocol described in *Methods In Malaria Research* (sixth edition),[24] 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 $MgCl_2$, 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 sedimented 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 $MgCl_2$ 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′-GCTATATATACGTAC-TGTGAACCCTACCAGACATGCA-CTG-3′/BIOT-5′-CAGTGCATGTCTGGTAGG-GTTCACAGTACGTAT-ATATAGC-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 5U 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[6] following the Peters 4 day suppressive test,[25] with slight modifications. Five CL57BL/6 mice per treatment group were infected intraperitoneally (i.p.) with 10^5 *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 nonequal 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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