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Lysine Acetylation in Sexual Stage Malaria Parasites Is a Target for Antimalarial Small Molecules

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Therapies to prevent transmission of malaria parasites to the mosquito vector are a vital part of the global malaria elimination agenda. Primaquine is currently the only drug with such activity; however, its use is limited by side effects. The development of transmission-blocking strategies requires an understanding of sexual stage malaria parasite (gametocyte) biology and the identification of new drug leads. Lysine acetylation is an important posttranslational modification involved in regulating eukaryotic gene expression and other essential processes. Interfering with this process with histone deacetylase (HDAC) inhibitors is a validated strategy for cancer and other diseases, including asexual stage malaria parasites. Here we confirm the expression of at least one HDAC protein in *Plasmodium falciparum* gametocytes and show that histone and nonhistone protein acetylation occurs in this life cycle stage. The activity of the canonical HDAC inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA; Vorinostat) and a panel of novel HDAC inhibitors on early/late-stage gametocytes and on gamete formation was examined. Several compounds displayed early/late-stage gametocytocidal activity, with TSA being the most potent (50% inhibitory concentration, 70 to 90 nM). In contrast, no inhibitory activity was observed in *P. falciparum* gametocyte exflagellation experiments. Gametocytocidal HDAC inhibitors caused hyperacetylation of gametocyte histones, consistent with a mode of action targeting HDAC activity. Our data identify HDAC inhibitors as being among a limited number of compounds that target both asexual and sexual stage malaria parasites, making them a potential new starting point for gametocytocidal drug leads and valuable tools for dissecting gametocyte biology.

Malaria remains a significant infectious disease, resulting in hundreds of millions of clinical cases and an estimated 0.8 million to 1.2 million deaths annually (1). The lack of a licensed malaria vaccine and the increasing incidence of parasite resistance to current drugs (2, 3) exacerbate the global burden of malaria and hinder efforts to eliminate this disease. Most current antimalarial drugs target intraerythrocytic asexual stage parasites, which are responsible for the clinical symptoms of malaria. Although the development of new drugs that target the asexual blood stage remains a high priority, the elimination of malaria also requires the development of drugs to target other key life cycle stages, including preerythrocytic (liver) and gametocyte stages. Targeting of preerythrocytic stage parasites has the potential for true causal prophylaxis, while targeting of gametocytes may block parasite transmission. *Plasmodium* gametocytes are the sexual stage of the malaria parasite that develops in red blood cells and can be transmitted directly from the mammalian host to the female *Anopheline* mosquito vector during a blood meal. Gametocytemia can persist for 2 to 8 weeks after asexual infection is cleared (4), depending on the drug used, allowing parasite transmission to continue. There are currently no licensed antimalarial drugs with a demonstrated ability in a clinical setting to kill or inhibit gametocytes that are safe for communitywide use. The only clinically used drug that can eliminate mature gametocytes, primaquine, is contraindicated for people with severe glucose-6-phosphate dehydrogenase (G6PD) deficiency. In these patients, primaquine treatment has the potential to result in hemolytic anemia. Primaquine is also considered unsafe during pregnancy, as the G6PD status of the fetus is not known.

During *Plasmodium falciparum* infection, gametocytes generally appear at about 10 to 14 days after the first appearance of asexual stage parasites in the host bloodstream. Gametocytogenesis, the process leading to the development of gametocytes from asexual stage parasites, represents a transition period during which time the parasite differentiates morphologically and biochemically. This maturation process can be divided into five distinct stages (stages I to V) on the basis of light and electron microscopy (5). Stage I and early stage II parasites are almost indistinguishable from asexual stage parasites. Parasites committed to sexual development become morphologically distinguishable only at late stage II and stage III, about 72 to 96 h after inva-

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sion of a committed asexual stage merozoite into an erythrocyte. As the gametocyte matures, it transitions through stage IV to stage V, where parasites have a characteristic crescent shape. Upon ingestion by a mosquito, male gamete formation occurs rapidly (~20 min) and is induced by a decrease in temperature and the mosquito-derived gametocyte-activating factor xanthurenic acid (6). This process involves three rounds of DNA replication and axoneme assembly, resulting in the release of up to eight gametes per male gametocyte (7, 8). In contrast, female gametocytes hold ~370 gene transcripts in translational repression (controlled by DOZI) until gamete formation, egress from the red blood cell, and fertilization (9).

The process of gametocytogenesis involves numerous genetic and metabolic changes that are not fully understood, but it is clear that these changes impact drug efficacy. Asexual blood and early gametocyte stages (stages I to III) have similar metabolic profiles, and consequently, many drugs are assumed to be active against both of these forms, although recent work suggests that there may be fewer cross-efficacious drugs than was first thought (10). In contrast, mature (stage IV and V) gametocytes have been thought to be relatively less metabolically active than asexual stage parasites and, with the exception of primaquine, have been considered insensitive to currently used antimalarial drugs. However, recent work has shown that rather than being largely quiescent in regard to their metabolic activities, gametocyte development is associated with more metabolic changes than was previously thought (11). This suggests that mature gametocytes do turn on the different proteins and pathways needed for rapid development into gametes. During gametocytogenesis and transmission, *P. falciparum* parasites express new sets of genes and proteins likely to be important for sexual development, survival, and reproduction in the mosquito vector (12–19). For example, gametocyte maturation and gametocytogenesis are accompanied by the coordinated expression of adhesive proteins, such as the epidermal growth factor domain-containing proteins *P. falciparum* s25 (*Pf*s25) and *P. falciparum* s28 (*Pf*s28), the 6-Cys proteins *P. falciparum* s230 (*Pf*s230) and *P. falciparum* s48/45 (*Pf*s48/45), and the LCCL domain-containing *P. falciparum* CCp (*Pf*CCp) proteins (20–23). Recent work has also identified the *P. falciparum* gametocyte development 1 (*Pfgdv1*) gene as being critical for gametocyte production (24). In addition, transcriptomic data demonstrate significant changes in a subset of genes immediately following malaria parasite transmission to the mosquito (17).

Radical changes in gene expression in response to different morphological states and environments is well documented for asexual blood-stage parasites, where epigenetic control is thought to contribute to the regulation of gene expression across the intraerythrocytic developmental cycle by maintaining *Plasmodium* genomic hetero- and euchromatin domains (25–29). Although no studies have yet been carried out on epigenetic control during gametocyte development, the observed changes in gene transcription and protein expression suggest a role for epigenetic control of gene expression during this life stage. This prompted us to investigate the effect of known chemical epigenetic modifiers, namely, histone (also called lysine) deacetylase (HDAC) inhibitors, on gametocytes with a view to identifying novel tools to study gametocyte development and new transmission-blocking approaches. HDACs are validated drug targets for asexual stage *P. falciparum* parasites (reviewed in reference 30), with potent antimalarial HDAC inhibitors causing alterations in global histone acetylation profiles and perturbing transcriptional control in asexual blood-stage parasites, causing both up- and downregulation of sets of genes (31–33). Little is known about *Plasmodium* HDACs in the sexual stage of reproduction of malarial parasites.

MATERIALS AND METHODS

Compounds used in this study. Suberoylanilide hydroxamic acid (SAHA; Sigma-Aldrich) was prepared as 10 to 200 mM stock solutions in 100% dimethyl sulfoxide (DMSO). *N*-[4-[3-[[[7-(Hydroxyamino)-7-oxoheptyl]amino]carbonyl]-5-isoxazolyl]phenyl]-1,1-dimethylethyl-ester and carbamic acid (CAY10603; Cayman Chemicals) were prepared as 10 mM stock solutions in 100% DMSO. Trichostatin A (TSA; Sigma-Aldrich) and chloroquine (chloroquine diphosphate salt; Sigma-Aldrich) were prepared as 10 to 50 mM stocks in ethanol and Milli-Q ultrapure water, respectively. Tosyllysine chloromethyl ketone hydrochloride (TLCK; Sigma-Aldrich) was prepared as a 3 mM stock in methanol. Details of chemical synthesis, including synthetic schemes, of hydroxamate-based HDAC inhibitors 1 to 5 (see Fig. 3 for structures) can be found in the supplemental material.

***P. falciparum* culture and gametocyte preparation.** Asexual stage *P. falciparum* drug-sensitive (line 3D7 [34]) and drug-resistant (line Dd2 [35]) parasites were cultured in type O-positive human erythrocytes in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated human serum and 50 mg/liter hypoxanthine, using a modified method of Trager and Jensen (1976) (36). Parasites were synchronized by sorbitol treatment, as previously described (37). Gametocytes were obtained using a modification of a published protocol (38) (method 1). Briefly, cultures of synchronous 3D7c (39) ring-stage parasites at 2 to 3% parasitemia were cultured through one invasion cycle to achieve a parasitemia of 6 to 8%. Following treatment with 5% sorbitol (37) to synchronize for ring stages, parasites were cultured until they reached the trophozoite stage and then diluted to 2% parasitemia and 5% hematocrit with fresh uninfected human erythrocytes. Following reinvasion to the ring stage (termed method 1, day 0 [M1-d0]), cultures were maintained under standard conditions with a daily medium change but without the addition of fresh erythrocytes. Daily medium changes included 5% sorbitol (40) or 50 mM *N*-acetylglucosamine (NAG) (41) to remove asexual stage parasites. Gametocyte maturation was monitored daily by microscopic examination of Giemsa-stained thin blood smears.

***P. falciparum* culture and gametocyte preparation for early and late stage gametocyte imaging-based viability assays.** *P. falciparum* NF54 parasites expressing the gametocyte-specific protein s16 (*Pf*s16) fused to green fluorescent protein (GFP) (NF54$^{Pfs16\text{-}GFP}$) were cultured and induced as described previously (42) (method 2). Briefly, sorbitol-synchronized asexual stage parasites were cultured at low parasitemia, and trophozoites were isolated on magnetically activated cell sorting (MACS) columns at day −3. The hematocrit was dropped from 5% to 1.25%. After overnight incubation, ring-stage parasites (10 to 15% parasitemia; day −2) received three parts fresh medium (with one part spent/used medium not being replaced) and were then left to develop into gametocyte-committed or noncommitted trophozoites through an overnight incubation. The day −1 trophozoite parasitemia was reduced to 3%, and the culture was incubated overnight. On day 0 (termed method 2, day 0 (M2-d0), young ring-stage parasites and spontaneously generated gametocytes were observed. Spontaneously generated gametocytes were removed from the ring-stage culture using MACS columns. For use in the early-stage (stage I to III) MitoTracker Red CM-H_2XRos imaging-based viability assay, parasitemia was reduced to 4% rings at 2.5% hematocrit, the culture was incubated overnight, and medium was replaced (day 1) before a further 24 h incubation. On day 2, early gametocytes were isolated from asexual ring-stage parasites using MACS columns. Day 0 parasites were also cultured in medium containing 50 mM NAG, with medium exchange being performed daily up to day 8 for use in the MitoTracker Red CM-H_2XRos late-stage (stage IV and V) gametocyte imaging-based viability assay.

Diagnostic RT-PCR. Total RNA was isolated from trophozoites of the non-gametocyte-producing strain F12 and different stages of the gametocyte-producing strain NF54 using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Stages isolated from the NF54 strain included enriched immature stage III gametocytes, nonactivated stage V gametocytes, and stage V gametocytes following 30 min activation with 100 μM xanthurenic acid. RNA preparations were treated with RNase-free DNase I (Qiagen) to remove genomic DNA contamination, followed by phenol-chloroform extraction and ethanol precipitation. cDNA synthesis was carried out using a SuperScript III first-strand synthesis system (Invitrogen), following the manufacturer's instructions. PCR was performed using the following conditions: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 1 min. The stage specificity of the RNA samples was verified by monitoring transcripts of two stage-specific markers: *P. falciparum* apical membrane antigen 1 (*Pf*AMA1) of asexual blood stages and the gametocyte-specific LCCL domain-containing protein *Pf*CCp2. Controls without reverse transcriptase (RT) were included to ensure that the samples were not contaminated with genomic DNA. PCR products were analyzed by agarose gel electrophoresis. The transcript of the ubiquitous metabolic protein *P. falciparum* fructose-1,6-bisphosphate aldolase (*Pf*Aldo) was used as a loading control. The primers used for diagnostic RT-PCR are shown in Table S1 in the supplemental material.

Immunofluorescence assay. Mature stage V 3D7c gametocytes (method 1) were cultured as described above and washed 3 times with RPMI 1640 medium (Life Technologies), and thin blood films were prepared on glass microscope slides. Slides were fixed with ice-cold 90% acetone–10% methanol and then probed using an anti-*P. falciparum* HDAC1 (anti-*Pf*HDAC1) polyclonal rabbit antibody in 3% bovine serum albumin (Bovogen). These antisera were raised (IVMS, South Australia. Australia) against a C-terminal peptide of *Pf*HDAC1 (PlasmoDB gene accession number PF3D7_0925700) with a previously published amino acid sequence (43). Specificity was confirmed via Western blotting against recombinant *Pf*HDAC1 (Sigma-Aldrich) (data not shown). Following washing to remove unbound primary antibody, slides were incubated with goat anti-rabbit IgG-Cy2 (Jackson) and Hoechst 33258 dye (Sigma-Aldrich) to stain the nuclei. Cells were visualized on an Applied Precision DeltaVision deconvolution microscope. Prebleed and secondary antibody-only controls were also included to ensure that the signal was specific.

Analysis of gametocyte lysine acetylation profiles. Gametocytes were generated from synchronous *P. falciparum* 3D7c asexual stage parasite cultures as described above (method 1), and samples were collected on days 4, 6, 8, and 10 relative to the start of induction (M1-d0). Protein lysates were prepared by pelleting the cells by centrifugation, lysing with 0.15% saponin (Sigma-Aldrich), and then washing extensively using phosphate-buffered saline (PBS; pH 7.4) to remove hemoglobin. Parasite pellets were then resuspended in 1× SDS-PAGE loading dye, heat denatured at 96°C for 3 min, and then separated via SDS-PAGE (~5×10^6 parasite equivalents per lane). For analysis of low-molecular-mass (<~25-kDa) histone proteins, 15% SDS-PAGE was used. For nonhistone protein analysis, 10% SDS-PAGE was used to allow separation and analysis of proteins of >~25 kDa. Protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Roche), and Western blotting was carried out using Odyssey blocking buffer (LI-COR Biosciences) according to the manufacturer's instructions. Anti-pan-acetyl lysine (K103) monoclonal primary antiserum (Cell Signaling Technology) was used with goat anti-rabbit IRDye secondary antisera (LI-COR Biosciences) to detect changes in nonhistone protein acetylation. Anti-tetra-acetylated (lysines 5, 8, 12, and 16) H4 histone primary antibody (Millipore) was used with goat anti-rabbit IRDye secondary antibody (LI-COR Biosciences) to detect changes in histone H4 acetylation. Antisera recognizing *Pf*s16 and *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase (*Pf*GAPDH) (16, 44, 45) were used as loading controls. *Pf*s16 is a gametocyte stage-specific protein (46), and GAPDH is a housekeeping protein present in *P. falciparum* asexual blood stages and in gametocytes. Membranes were imaged using an Odyssey infrared imaging system (LI-COR Biosciences).

Asexual stage *P. falciparum in vitro* growth inhibition assays. The *in vitro* activity of compounds against asexual *P. falciparum* lines 3D7 and Dd2 was determined using [^{3}H]hypoxanthine incorporation (47, 48). Serial dilutions of compounds were prepared in culture medium, and then synchronous ring-stage-infected erythrocytes were added at 0.25% parasitemia and a 2.5% final hematocrit. Following 48 h of incubation, [^{3}H]hypoxanthine (0.5 μCi/well) was added to each well and the cultures were incubated for a further 24 h. Hypoxanthine incorporation into parasites was measured by harvesting onto 1450 MicroBeta filter mats (Wallac) and counting using a 1450 MicroBeta liquid scintillation counter. The percent inhibition of growth compared to the growth of the matched DMSO controls (0.5%) was determined. Each assay was carried out in triplicate wells on three separate occasions. In each assay, chloroquine was included as an internal control. The 50% inhibitory concentrations (IC_{50}s) were determined using log-linear interpolation of inhibition curves (49) and are presented as the means ± standard deviations (SDs) of the three independent assays.

***In vitro* mammalian cell toxicity assays.** Cytotoxicity assays were performed essentially as previously described (50). Briefly, neonatal foreskin fibroblasts (NFFs) were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (CSL Biosciences) and 1% penicillin-streptomycin (Life Technologies). Cells were seeded into wells of 96-well tissue culture plates (3,000 cells per well) and cultured for 24 h at 37°C in 5% CO_2 before being treated with a dilution series of each compound. After 72 h, medium was removed and plates were washed in PBS. Cells were fixed with denatured alcohol before a water wash and the addition of sulforhodamine B (0.4%; 50 μl; Sigma-Aldrich). After staining for 1 h, plates were washed three times with 1% acetic acid, and then 100 μl of 10 mM Tris base (unbuffered, pH >9) was added to each well. Plates were read at 564 nm in an enzyme-linked immunosorbent assay microplate reader. Percent inhibition of growth compared to the growth of the matched DMSO controls (0.5%) was then determined. Each assay was performed in triplicate on three separate occasions, and chloroquine was included as an internal control in each assay. IC_{50}s were determined using log-linear interpolation of inhibition curves (49) and are presented as means ± SDs of the three independent assays.

Gametocyte ATP assay. Mature stage V gametocytes (method 1) were harvested at days 10 to 12 relative to the start of induction (M1-d0) and enriched by magnetic separation using MACS columns (Miltenyi Biotec) (38). Testing was performed using cryopreserved and thawed gametocytes essentially as previously described (51). Approximately 5,000 thawed gametocytes were dispensed into the wells of a 96-well plate with test compounds or controls to give a final volume of 100 μl, and the plate was incubated at 37°C for 24 or 48 h under standard culture conditions. BacTiter-Glo reagents (G8231; Promega) were added to a final volume of 200 μl, and the assay was read using a GloMax 96 microplate luminometer with an integration constant of 0.5 s. Sample readouts were compared to the control ATP expression levels, and the percent ATP production was calculated. A nonparametric two-tailed Student's *t* test was used for statistical analysis of the difference between the test compounds and the controls. All compounds were dissolved in DMSO and tested in triplicate on three separate occasions at a concentration of 5 μM (0.5% DMSO). The assay included a vehicle control of standard culture medium with 0.5% DMSO (no drug). Methylene blue (5 μM) was included as a positive control.

MitoTracker Red CM-H_2XRos late-stage gametocyte imaging-based viability assay. Highly synchronous stage IV gametocytes (method 2) were produced from transgenic NF54 *P. falciparum* parasites expressing the gametocyte-specific protein *Pf*s16 fused to green fluorescent protein (NF54$^{Pfs16\text{-}GFP}$) (42, 52). Gametocytes were harvested using MACS columns (Miltenyi Biotec) on day 8 relative to the start of induction (M2-d0) and added to 384-well imaging plates at 33,000 gametocytes per well. After addition of test or control compounds, the cells were incubated for

72 h at 37°C in 5% CO_2, 5% O_2, and 80% N_2. MitoTracker Red CM-H_2XRos (Invitrogen) was then added to each well (0.07 μg/ml) and the cells were incubated for 12 to 18 h, as described above. Compound vehicle alone (0.4% DMSO) and puromycin (5 μM) were used as controls. Gametocyte viability was evaluated on an Opera (PerkinElmer) high-content screening system. The images acquired for GFP and MitoTracker Red CM-H_2XRos were overlaid, and the number of viable elongated gametocytes per image was determined using an algorithm based on Acapella software developed for use with the Opera imaging system. Compound data were normalized to the relative percent inhibition of the controls (5 μM puromycin and 0.4% DMSO). IC_{50} calculation was performed using the GraphPad Prism program (version 4.0). Data were analyzed using 4-parameter nonlinear regression (curve fit) with the sigmoidal log dose-response (variable slope). Each compound was tested in duplicate at 16 concentrations in at least two independent experiments. A nonparametric two-tailed Student's *t* test was used for statistical analysis of the difference between the test compounds and the controls.

MitoTracker Red CM-H_2XRos early-stage gametocyte imaging-based viability assay. Highly synchronous early-stage gametocytes were isolated (method 2) on day 2 relative to the start of induction (M2-d0) using MACS columns and added to 384-well imaging plates at 33,000 gametocytes per well. The remainder of the assay was consistent with the MitoTracker Red CM-H_2XRos late-stage gametocyte imaging-based viability assay (42).

Assessment of gametocyte development via microscopy. Stage III and IV gametocytes were seeded into tissue culture plates at ~0.5% gametocytemia and 5% hematocrit with test compounds (5 μM) or the DMSO vehicle control (0.05%). Chloroquine was used as a negative control. For microscopic evaluation of gametocytemia (number of gametocytes per 100 erythrocytes), Giemsa-stained thin blood films were examined via light microscopy. Approximately 3,000 to 4,000 cells were counted in each assay. Results of two independent experiments were pooled, and data are presented as the mean percent gametocytemia ± SD compared to that for the DMSO vehicle-treated control cultures (taken as 100%). A nonparametric two-tailed Student's *t* test was used for statistical analysis of the difference between the test compounds and the controls.

***P. falciparum* exflagellation inhibition assay.** Exflagellation assays were carried out according to previously published methods (53–55). A total volume of 100 μl of mature NF54 gametocyte culture was preincubated with SAHA and TSA at concentrations ranging from 0.125 to 2 mM and 0.06 to 0.5 mM, respectively, for 15 min at 37°C. Samples were then transferred to room temperature, and xanthurenic acid was added at a concentration of 100 μM for activation. After 15 min, the number of exflagellation centers in 30 optical fields was counted, in duplicate, using a Leica DMLS microscope at 400-fold magnification. Two independent experiments were performed for SAHA, and three independent experiments were performed for TSA. The inhibition of exflagellation was calculated as a percentage of the number of exflagellation centers in compound-treated cultures in relation to the number for the untreated controls (the value for untreated controls was set to 100%). TLCK at a concentration of 30 μM was used as a positive control. The IC_{50}s were calculated from variable-slope sigmoidal dose-response curves using the GraphPad Prism program (version 5).

Ookinete conversion assay. Ookinete conversion assays were performed using *Plasmodium berghei* ANKA parasites constitutively expressing GFP and followed existing protocols (56, 57), modified to utilize 48-well culture plates. All inhibitors were dissolved in DMSO, and the results were compared to those of the control treatments with 0.1% DMSO alone. Fluorescent ookinetes were counted in duplicate for each experiment.

Hyperacetylation assay. Hyperacetylation assays were carried out by incubating stage III and IV *P. falciparum* 3D7c gametocytes for 6 h under standard *in vitro* culture conditions with different concentrations of test compound or the vehicle control (0.05% DMSO). Protein lysates were prepared as described above for asexual stage parasites. Briefly, cells were harvested by centrifugation, lysed with 0.15% saponin (Sigma-Aldrich), and washed extensively using PBS (pH 7.4) to remove hemoglobin. Pellets were resuspended in 1× SDS-PAGE loading dye, heat denatured at 96°C for 3 min, and then separated via 15% SDS-PAGE. Protein was transferred to a PVDF membrane (Roche), and Western blotting was carried out using Odyssey blocking buffer (LI-COR Biosciences) according to the manufacturer's instructions. The following antisera were used to detect acetylated histones: anti-tetra-acetylated H4 (lysines 5, 8, 12, and 16), anti-acetylated H3 (N terminus), and anti-acetylated H3 (lysine 9) (Millipore). Antisera recognizing *Pf*s16 and *Pf*GAPDH (44, 45) were used as loading controls. Membranes were imaged using an Odyssey infrared imaging system (LI-COR Biosciences).

RESULTS

HDACs are expressed in *P. falciparum* gametocytes. The genome of *P. falciparum* encodes five HDAC proteins, termed *Pf*HDAC1 to *Pf*HDAC3 (PlasmoDB gene accession numbers PF3D7_0925700, PF3D7_1472200, and PF3D7_1008000, respectively), *Pf*Sir2a (PlasmoDB gene accession number PF3D7_1328800), and *Pf*Sir2b (PlasmoDB gene accession number PF3D7_1451400). Previous transcriptomic and proteomic studies have indicated that some *Pf*HDACs are expressed in gametocyte-stage parasites (12, 16), with *Pf*HDAC1 shown by Northern blotting to be transcribed in gametocytes (43). Here we used diagnostic RT-PCR to confirm the transcription of all five HDACs in early gametocytes (stage III), late gametocytes (stage V), and stage V gametocytes following 30 min activation with 100 μM xanthurenic acid (Fig. 1a). The stage specificity of the RNA samples was confirmed by examining the transcription of the asexual blood-stage-specific *Pfama1* gene and the gametocyte-specific *Pfccp2* gene. Transcription of *Pfaldo*, which encodes the ubiquitous plasmodial fructose-1,6-bisphosphate aldolase, was used as a loading control. Samples lacking reverse transcriptase were used to demonstrate that the RNA samples were devoid of genomic DNA (Fig. 1a).

Next, the expression of *Pf*HDAC1, a validated target of antimalarial HDAC inhibitors in asexual stage parasites (58), was confirmed in *P. falciparum* gametocytes by Western blotting and immunofluorescence assay using a polyclonal antiserum raised against a C-terminal peptide (43) of *Pf*HDAC1 (Fig. 1b and c). For Western blotting, protein lysates were prepared from asexual stage *P. falciparum* 3D7 trophozoites and 3D7c gametocytes (method 1) on days 4, 6, 8, and 10 relative to the start of induction (M1-d0). Microscopic examination of Giemsa-stained thin blood films (Fig. 2a) showed that cultures harvested on day 4 contained 83.5% ± 3.5% gametocyte stages I to III, with ~16.5% of parasites not being able to be distinguished from asexual stages. On day 6, cultures were 95% ± 2.8% gametocytes (stages III and IV), and on days 8 and 10, no asexual blood stages were observed (100% gametocytes; stages IV and V). Two-color Western blots showed a band of ~50 kDa in all samples when reacted with anti-*Pf*HDAC1 rabbit antiserum; this corresponds to the predicted size of *Pf*HDAC1. As expected, an ~16-kDa band was present in induced gametocyte samples but not asexual stage 3D7 parasite samples, using mouse antiserum specific to the gametocyte marker protein *Pf*s16 (Fig. 1b). Immunofluorescence assay data using anti-*Pf*HDAC1 showed localization primarily to the nucleus of late-stage gametocytes (Fig. 1c), consistent with the asexual blood stage localization of this protein (43). Together these data confirm that *Pf*HDAC1 is expressed in both asexual and gametocyte stages.

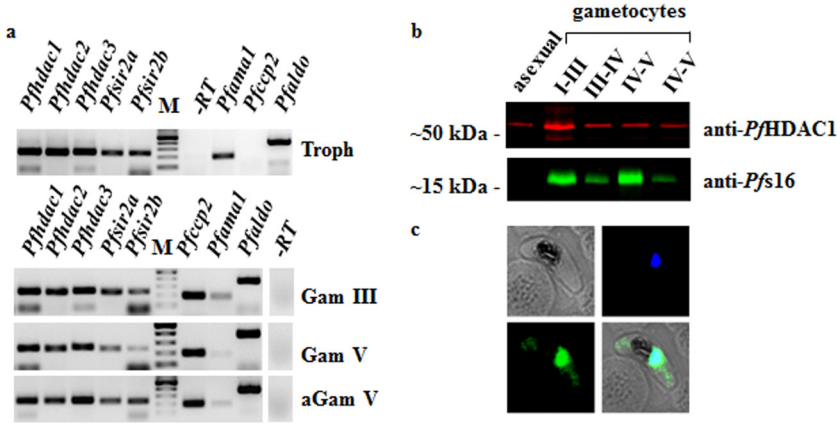

FIG 1 Expression of HDACs in *P. falciparum* gametocytes. (a) Diagnostic RT-PCR on transcripts from trophozoites (Troph) of gametocyte-less strain F12, purified NF54 stage III and V gametocytes (Gam III and Gam V, respectively), and stage V gametocytes at 30 min postactivation (aGam V) with 100 μM xanthurenic acid at room temperature. RT-PCR was carried out with *Pf*HDAC-specific primers. The expression levels of *Pfama1* and *Pfccp2* were used to verify blood-stage and gametocyte-specific expression, respectively. Samples lacking reverse transcriptase (−RT) were used as controls for genomic DNA contamination. The fructose-1,6-bisphosphate aldolase-encoding gene (*Pfaldo*) served as a loading control. All PCR products had nucleotide lengths of 200 to 300 bp. Lane M, 100-bp marker. (b) Western blot analysis of protein lysates from trophozoite-stage 3D7 *P. falciparum* parasites (asexual) and *P. falciparum* 3D7c gametocytes (method 1) on days 4 (stages I to III), 6 (stages III and IV), 8 (stages IV and V), and 10 (stages IV and V) relative to the start of induction (M1-d0). Protein lysates were analyzed by SDS-PAGE and two-color Western blot analysis using an Odyssey infrared imaging system (LI-COR Biosciences). The same membrane was probed with anti-*Pf*HDAC1 rabbit antiserum (red) and anti-*Pf*s16 mouse antiserum (green). (c) Immunofluorescence image of late-stage gametocytes showing a bright-field image (top left), nuclear staining with DAPI (4′,6-diamidino-2-phenylindole; top right), predominantly nuclear localization with anti-*Pf*HDAC1 antiserum (bottom left), and an overlay (bottom right).

Histone and nonhistone proteins are acetylated in different gametocyte stages. The acetylation of histone and nonhistone proteins was examined in gametocytes using Western blotting with antisera that recognize tetra-acetylated histone H4 and pan-acetylated lysine residues. A similar profile of tetra-acetylated histone H4 reactivity was observed in *P. falciparum* 3D7c protein lysates as gametocytes developed (Fig. 2b). As previously observed for asexual blood-stage parasites (59), Western analysis using antiserum that pan-specifically reacts with all acetylated lysine residues detected multiple protein bands spanning a wide mass range. The most strongly reactive band at ~60 kDa was found to alter as parasites progressed from early- to late-stage gametocytes. The acetylation of other proteins remained constant from days 4 to 10 relative to the start of induction (M1-d0), indicating that the reduced acetylation reactivity seen on days 6 and 8 using the pan-acetyl lysine antiserum was not due to unequal loading.

Gametocytes are inhibited *in vitro* by HDAC inhibitor exposure. To assess the susceptibility of gametocytes to chemical epigenetic modifiers, the effect of three commercially available HDAC inhibitors was first examined. TSA and SAHA (Fig. 3) are canonical paninhibitors that act on class I/II eukaryotic HDACs 1 to 9 with roughly equal potency (60) and have previously been

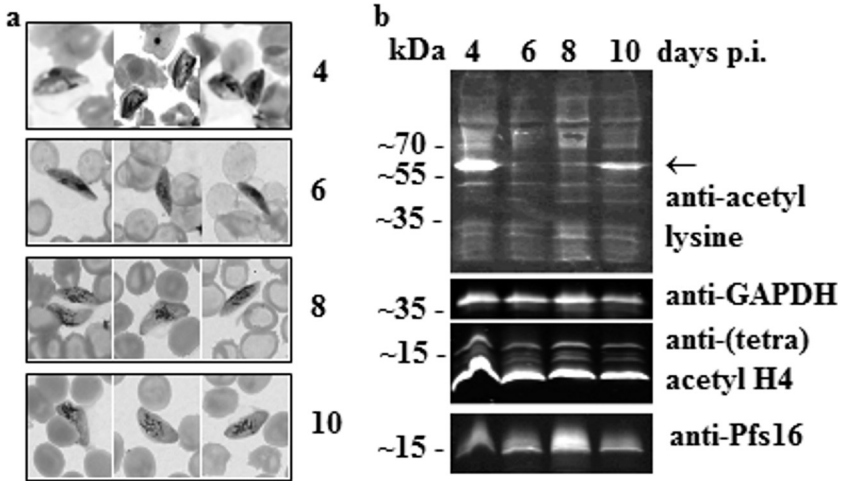

FIG 2 Lysine acetylation during gametocyte development. (a) Samples were collected from *P. falciparum* 3D7c parasites (method 1) on days 4 (stages I to III), 6 (stages III and IV), 8 (stages IV and V), and 10 (stages IV and V) relative to the start of gametocyte induction (M1-d0). (b) Protein lysates were analyzed by SDS-PAGE and two-color Western blot analysis using an Odyssey infrared imaging system (LI-COR Biosciences). Membranes were probed with anti-pan-acetyl lysine (K103) mouse antiserum and anti-*Pf*GAPDH rabbit antiserum or anti-tetra-acetylated (lysines 5, 8, 12, and 16) histone H4 rabbit antiserum and anti-*Pf*s16 mouse antiserum. Arrow, an ~60-kDa protein showing altered acetylation during gametocyte development. p.i., postinduction.

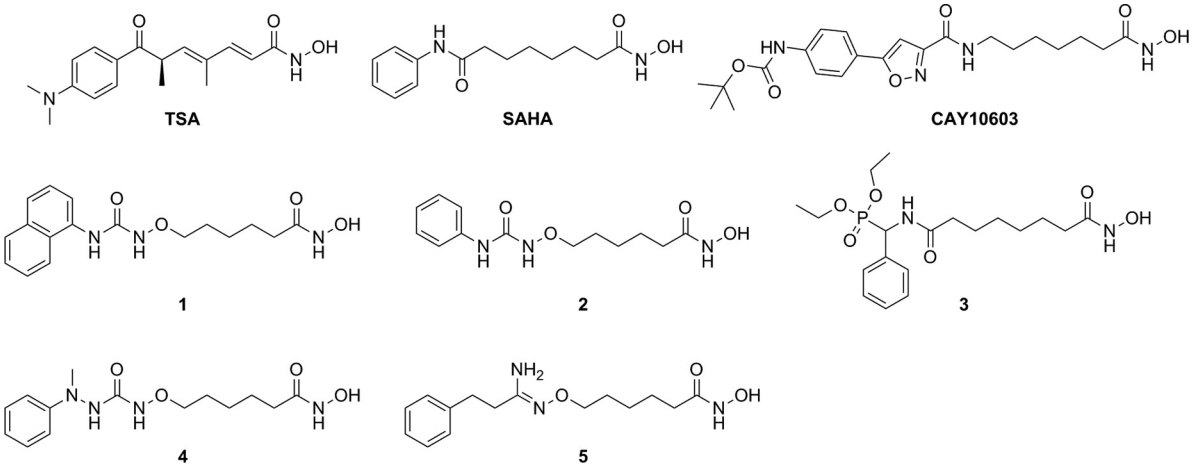

FIG 3 Structures of the HDAC inhibitors used in this study.

shown to have potent inhibitory activity against asexual blood-stage *P. falciparum* parasites (Table 1) (61–63). The third HDAC inhibitor tested, CAY10603 (Fig. 3), while also having a hydroxamate zinc-binding group like TSA and SAHA, demonstrates some isoform selectivity for human HDAC6 (64). When we tested the activity of this compound against asexual stage *P. falciparum* parasites (Table 1), we observed a level of inhibitory activity similar to that of SAHA against *P. falciparum* drug-sensitive (3D7 line; IC_{50}, 0.17 μM) and drug-resistant (Dd2 line; IC_{50}, 0.34 μM) parasites.

To determine whether SAHA, TSA, and CAY10603 had activity against sexual stage malaria parasites, the compounds were tested against late-stage gametocytes using two previously published assays: an ATP production assay using 3D7c gametocytes (51) and an imaging-based viability assay that utilizes transgenic NF54 parasites expressing *Pfs*16 fused to the GFP reporter ($NF54^{Pfs16\text{-}GFP}$) combined with MitoTracker Red CM-H_2XRos staining (42). In each assay, methylene blue, which is a potent inhibitor of gametocyte development across all stages (52), was used as a positive control. The data demonstrate that exposure to 5 μM SAHA, CAY10603, or methylene blue for 48 h (Fig. 4a) significantly reduced gametocyte ATP activity ($P < 0.001$). Although there was a trend toward reduced ATP production in the presence of TSA, this effect was not statistically significant ($P > 0.05$). In contrast, the imaging-based viability assay showed a significant reduction ($P < 0.001$) in gametocyte viability for TSA, SAHA, and methylene blue, but not CAY10603 (Fig. 4b). To confirm these findings, microscopy was also used to monitor the HDAC inhibitor effect on *P. falciparum* gametocytes. All three HDAC inhibitors, but not the control compound, chloroquine, were found to significantly inhibit the growth/maturation of stage III and IV 3D7c gametocytes after 5 days of exposure (Fig. 4c and d).

Gametocytocidal activity of novel HDAC inhibitor analogues. To begin to assess the potential of HDAC inhibitors as new

TABLE 1 *In vitro* antimalarial activity of HDAC inhibitors against asexual and gametocyte-stage *P. falciparum* parasites

Compound	IC_{50} (μM)					
	Asexual stage[a]		Gametocyte[b]			
	3D7	Dd2	Early stage	Late stage	NFFs	Selectivity index[c]
TSA	0.01[d]	0.008[e]	0.09 ± 0.01	0.07 ± 0.02	0.2[d]	18–25 (3)
SAHA	0.12 ± 0.04[e]	0.19 ± 0.01[e]	1.41 ± 0.13	0.81 ± 0.21	4.90 ± 1.24	26–41 (6)
CAY10603	0.17 ± 0.03	0.34 ± 0.29	>4.00	>4.00	4.04 ± 0.74	12–24 (<1)
1	0.25 ± 0.07	0.32 ± 0.10	2.25 ± 0.67	2.12 ± 0.44	19.5 ± 3.8	36–78 (9)
2	0.45 ± 0.05	0.54 ± 0.31	6.65 ± 1.04	1.68 ± 0.33	11.4 ± 0.2	21–25 (2–7)
3	0.82 ± 0.09	1.32 ± 0.01	14.60 ± 2.36	3.20 ± 1.92	33.2 ± 9.1	25–40 (2–10)
4	1.09 ± 0.27	1.17 ± 0.16	>10.00	3.57 ± 0.29	37.1 ± 0.2	32–34 (3–10)
5	1.42 ± 0.60	3.13 ± 0.33	>10.00	9.15 ± 3.11	79.9 ± 12.0	26–56 (8–9)
MB[f]	ND[g]	ND	0.24 ± 0.02	0.23 ± 0.02	ND	ND
CQ[h]	0.01 ± 0.003	0.13 ± 0.02	0.20 ± 0.02	>120.00	21.4 ± 3.1	164–2,140 (<1)

[a] Results are the means of three independent *in vitro* [^{3}H]hypoxanthine growth inhibition assays, each of which was carried out in triplicate.
[b] Results are means ± SDs of 2 to 4 MitoTracker Red CM-H_2XRos gametocyte image-based viability experiments, each of which was carried out in duplicate.
[c] Selectivity indices are for asexual stage parasites and late-stage gametocytes (in parentheses) and were calculated as the mammalian cell IC_{50}/*P. falciparum* IC_{50}; larger values indicate greater parasite selectivity.
[d] Data previously reported (61, 76).
[e] Data previously reported (73).
[f] MB, methylene blue.
[g] ND, not determined.
[h] CQ, chloroquine.

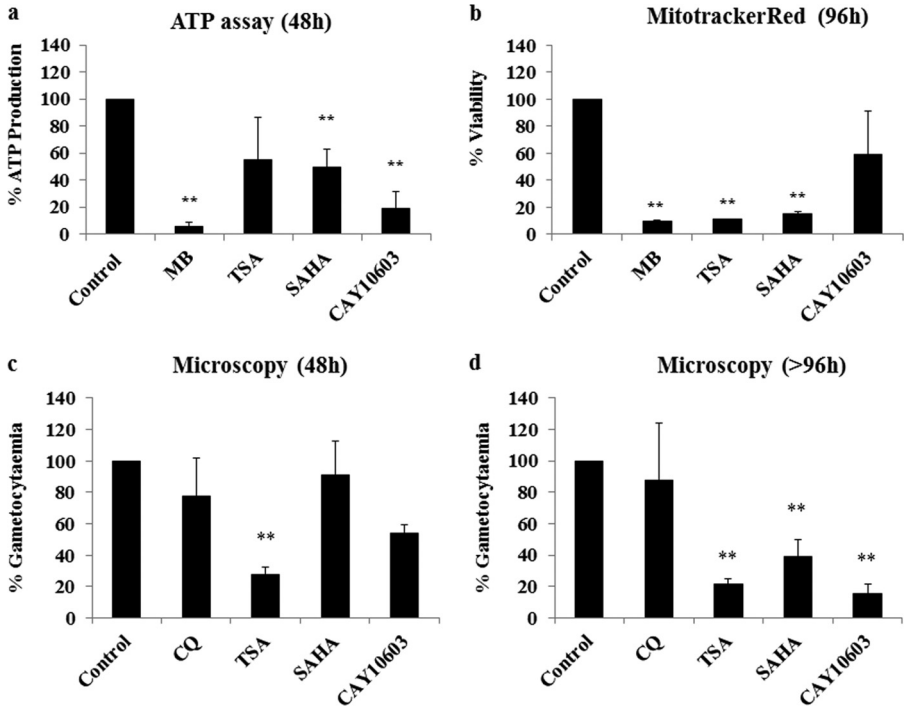

FIG 4 Activity of TSA, SAHA, and CAY10603 against gametocytes. (a) ATP activity assay with cryopreserved and thawed MACS-purified stage V 3D7c gametocytes following 48 h of exposure to 10 μM methylene blue (MB) or 5 μM TSA, SAHA, or CAY10603. Controls contained vehicle only (0.5% DMSO). The mean percent ATP production ± SD is shown for three independent experiments, each of which was carried out in triplicate wells (**, $P < 0.001$). (b) MitoTracker Red CM-H_2XRos imaging-based viability assay with stage IV and V NF45$^{Pfs16\text{-}GFP}$ gametocytes. Cells were incubated for 72 h with 4 μM methylene blue, TSA, SAHA, or CAY10603 and then incubated for 16 h with MitoTracker Red CM-H_2XRos (and compound) before imaging. Controls contained vehicle only (0.5% DMSO). The mean percent growth ± SD is shown for two independent experiments, each of which was carried out in duplicate wells (**, $P < 0.001$). (c and d) Stage III and IV gametocytes (~0.5% gametocytemia, 5% hematocrit) were cultured with 5 μM TSA, SAHA, CAY10603, or the antimalarial drug chloroquine (CQ) for 2 days (c) or 5 days (d), with medium (without compound) being changed on day 2. Control cultures were incubated in the same way but contained vehicle only (0.05% DMSO). Thin blood films were prepared and stained with Giemsa, and the number of gametocytes per 100 cells (percent gametocytemia) was determined by microscopy (>3,000 cells were counted). The mean percent gametocytemia ± SD compared to that for the vehicle controls (taken as 100%) from two independent experiments is shown (**, $P < 0.001$).

agents for blocking the transmission of gametocytes to the mosquito, we used the MitoTracker Red CM-H_2XRos imaging-based viability assay to compare the early- and late-stage gametocytocidal activity of TSA, SAHA, and CAY10603 with that of a panel of five other HDAC inhibitors (compounds 1 to 5; Fig. 3). The alkoxyurea compounds (compounds 1 and 2) were previously identified to be potent human pan-HDAC inhibitors (65). The phosphonic ester (compound 3), the alkoxysemicarbazide (compound 4), and the amidoxime derivative (compound 5) have not been described or tested before. The MitoTracker Red CM-H_2XRos imaging-based viability assay was selected because of the longer time of exposure to compounds (which ensures that the activities of slower-acting compounds are also captured), and in addition, it utilizes fresh versus cryopreserved gametocytes, thereby bypassing the liabilities associated with the freeze-thaw process. This assay also allowed direct comparisons between early- and late-stage gametocyte viability, as recently validated for high-throughput applications (10, 42).

As compounds 1 to 5 had not previously been tested for activity against asexual stage parasites, all five were first tested against chloroquine-sensitive and -resistant asexual stage *P. falciparum* parasites using the *in vitro* [^{3}H]hypoxanthine growth inhibition assay. Cytotoxicity against a normal human cell line was assessed using the sulforhodamine B *in vitro* cell toxicity assay (Table 1). Compounds 1 to 5 had asexual blood-stage activities with IC_{50}s ranging from 0.25 to 1.42 μM. The most active compounds were alkoxyurea compounds 1 (IC_{50}, 0.25 μM) and 2 (IC_{50}, 0.45 μM), while the alkoxysemicarbazide (compound 4; IC_{50}, 1.09 μM) and the amidoxime derivative (compound 5; IC_{50}, 1.42 μM) displayed considerably less potent inhibitory activity against the asexual blood stage *in vitro*. All compounds of this series were less active than the reference compounds, TSA and SAHA. The ability of one of the more potent compounds (Table 1, compound 1) to cause hyperacetylation of asexual blood-stage *P. falciparum* histones was demonstrated using trophozoite-stage parasites (see Fig. S1 in the supplemental material), confirming an effect on *P. falciparum* HDAC activity in asexual stage parasites. Comparison of the IC_{50}s for asexual blood-stage *P. falciparum* with those obtained for the normal NFF mammalian cell line shows that members of this panel of HDAC inhibitors have somewhat better selectivities (selectivity indices, 21 to 78) than SAHA or TSA (selectivity indices, 18 to 41) (Table 1).

When evaluated against early- and late-stage NF54$^{Pfs16\text{-}GFP}$ gametocytes, TSA was found to be the most potent compound, with IC_{50}s of 0.09 and 0.07 μM, respectively (Table 1). This activity was better than that observed for the control compound, methylene blue (IC_{50}, ~0.2 μM; Table 1). SAHA had less potent activity against early- and late-stage gametocytes, with IC_{50}s of 1.4 and 0.8

μM, respectively, while CAY10603 had an IC_{50} of >4 μM against both stages (Table 1). All five new HDAC inhibitor analogues displayed only modest gametocytocidal activity, with IC_{50}s ranging from 1.7 to >10 μM. As for the asexual stages, alkoxyurea compounds 1 and 2 showed the most potent inhibitory activity. The selectivity of these compounds for gametocytes versus mammalian cells was similar to that of TSA and SAHA (selectivity indices, 2 to 10; Table 1).

HDAC inhibitors are not effective in inhibiting gametocyte exflagellation. To assess the effect of HDAC inhibitors on male gamete formation, we carried out *in vitro* exflagellation experiments using mature *P. falciparum* NF54 gametocytes. During this process, exflagellating male gametes attach to nearby erythrocytes, thereby forming exflagellation centers, which can easily be observed under a microscope. Both SAHA and TSA inhibited exflagellation, but low-mM concentrations were required (SAHA IC_{50}, 0.50 ± 0.003 mM; TSA IC_{50}, 0.22 ± 0.04 mM; see Fig. S2 in the supplemental material). Tosyllysine chloromethyl ketone hydrochloride (TLCK), an inhibitor of trypsin-like serine proteases, was used as a positive control. TLCK was previously shown to effectively inhibit exflagellation (53, 54), and at a concentration of 30 μM, it fully blocked the formation of exflagellation centers.

A similar finding was also observed for the *in vitro* conversion of *P. berghei* mouse malaria gametocytes to ookinetes *in vitro*. This assay is a relatively robust predictor of transmission blocking in the insect stages of the parasite life cycle (66), providing information about the effects of compounds on gamete formation, fertilization, and ookinete formation. Treatment with SAHA at concentrations up to 25 μM had no effect on ookinete conversion. Similarly, TSA at 5 μM and CAY10603 at 2.5 μM were not inhibitory. In contrast, 1 μM cycloheximide completely inhibited ookinete formation. This suggests that HDAC inhibitors of this class have little impact on the sexual reproduction and transformation of the zygote to ookinete stages.

HDAC inhibitors cause hyperacetylation of *P. falciparum* gametocyte histone proteins. To determine whether HDAC inhibitors alter the lysine acetylation profile of proteins in gametocyte stages, hyperacetylation assays were carried out. Mixed stage III and IV gametocytes were treated for 6 h with 5 μM TSA, SAHA, CAY10603, or the control compound methylene blue or chloroquine. Protein lysates were analyzed by Western blotting using antisera specific for acetylated forms of histones H3 and H4 (Fig. 5) or using pan-acetyl lysine antiserum (see Fig. S3 in the supplemental material). In comparison to the vehicle control (0.05% DMSO)-, methylene blue-, or chloroquine-treated parasites, hyperacetylation was observed for all three HDAC inhibitors using antisera to tetra-acetylated H4 (lysines 5, 8, 12, and 16), N-terminally acetylated H3, and lysine 9-acetylated H3 (Fig. 5). In contrast, HDAC inhibitor treatment did not appear to alter the acetylation profile when nonhistone proteins were examined using the pan-acetyl lysine antiserum (see File S3 in the supplemental material).

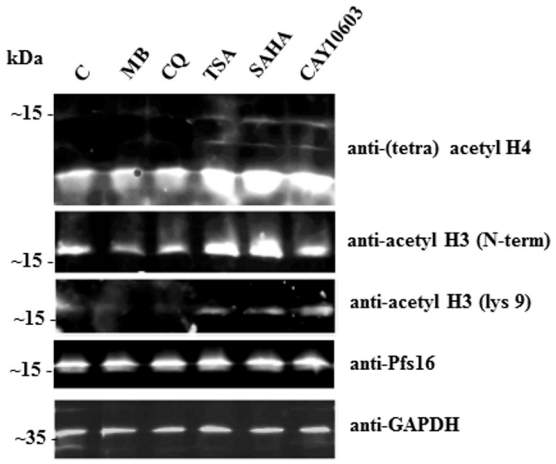

FIG 5 HDAC inhibitors cause hyperacetylation of gametocyte histones. Stage III and IV *P. falciparum* 3D7c gametocytes were treated with 5 μM methylene blue (MB), chloroquine (CQ), TSA, SAHA, CAY10603, or vehicle only (0.05% DMSO as a control [C]) for 6 h. Protein lysates were analyzed by SDS-PAGE and Western blotting using antisera recognizing acetylated forms of histone H3 and H4: anti-tetra-acetylated H4 (lysines 5, 8, 12, and 16), anti-acetylated H3 (N terminus [N-term]), and anti-acetylated H3 (lysine 9 [lys 9]). Antisera recognizing *Pfs*16 and *Pf*GAPDH were used as loading controls.

DISCUSSION

The eradication of malaria will require a multipronged approach, including the development of new therapies that block the transmission of malaria parasites from the human host to the mosquito vector (67). A major bottleneck in developing such transmission-blocking agents is our relatively poor understanding of how sexual stage malaria parasites develop within the host erythrocyte and which enzymes and proteins are essential for this process. Recent progress toward the *in vitro* production of large-scale, pure, gametocyte cultures (68) has now opened up new opportunities to improve our understanding of gametocyte biology and use this information to develop new drugs that target this malaria parasite life cycle stage. In this study, we examined lysine acetylation as a potential new therapeutic target for gametocytes. We confirm that at least one *P. falciparum* histone deacetylase (*Pf*HDAC) protein is expressed in gametocytes, that histone and nonhistone protein acetylation occurs in this life cycle stage, and that small molecules of the HDAC inhibitor class may be novel leads for blocking transmission of sexual stage malaria parasites to the mosquito vector.

HDACs, enzymes involved in regulating lysine acetylation of histone and nonhistone proteins in eukaryotic cells, are a recognized drug target in asexual stage malaria parasites (30, 58, 69). Five HDAC-encoding genes have been identified in the *P. falciparum* genome (70). While the two class III HDAC homologues, *Pf*Sir2a (PlasmoDB gene accession number PF3D7_1328800) and *Pf*Sir2b (PlasmoDB gene accession number PF3D7_1451400), are not essential to asexual stage *P. falciparum* survival *in vitro* (71, 72), the three class I and II HDAC homologues, *Pf*HDAC1 to *Pf*HDAC3 (PlasmoDB gene accession numbers PF3D7_0925700, PF3D7_1472200, and PF3D7_1008000, respectively), are potential targets of antimalarial HDAC inhibitors. *Pf*HDAC1 has >50% homology to other mammalian HDACs (43), and a recombinant form of this protein can be potently inhibited by antimalarial HDAC inhibitors like TSA and SAHA (58). In contrast, *Pf*HDAC2 and *Pf*HDAC3 share very little sequence homology with any mammalian HDACs and have not been characterized. Despite work demonstrating the potential of HDAC inhibitors against asexual *P. falciparum* parasites (recently reviewed in reference 30) and transcriptomic and proteomic data showing that *Pf*HDACs are expressed in both asexual stage and gametocyte-stage *P. falciparum* parasites (12, 15, 18, 43), this class of important transcriptional regulators has not previously been studied in gametocytes

or investigated as a target for blocking malaria parasite transmission.

Previous work from our group and others using antisera that specifically recognize acetylated forms of proteins has shown that both histone and nonhistone proteins are acetylated in asexual stage *P. falciparum* parasites (59, 73). When we used this approach to examine protein acetylation during gametocyte development, we found that while the tetra-acetylated H4 profile was similar in all developmental stages as gametocytes matured, the panacetylated lysine profile was altered. An ~60-kDa acetylated protein species was apparent in some, but not all, developmental stages. While this raises the intriguing possibility that this protein may play a role in gametocyte development, further work will be needed to identify this protein and characterize its precise role during the gametocyte life cycle. Likewise, our data showing histone H4 tetra-acetylation in all the development stages examined provides only a snapshot of histone acetylation in gametocytes. Further analysis of different acetylated forms of H4 and other histones is needed to improve our understanding of the role of lysine acetylation during gametocyte development.

Given that our data showed that lysine acetylation occurs in gametocytes (Fig. 2) and confirmation that at least one *Pf*HDAC is expressed in this life cycle stage (Fig. 1), we next examined the gametocytocidal activity of three commercially available HDAC inhibitors (TSA, SAHA, and CAY10603) using different published assays: an ATP metabolic assay, an imaging-based viability assay, and assessment via microscopy (Fig. 4). Overall, the three methods gave a similar trend, with all three HDAC inhibitors demonstrating some gametocytocidal activity, although there were variations in the levels of inhibition obtained. While it is not possible to directly compare the data obtained for the different assay methods, given that factors such as variations in gametocyte induction and purification protocols for the different assays, stage- and sex-specific ratio differences, variations in the length of each assay, and different endpoint detection methods preclude this, we were able to illustrate a general trend. Each of these factors would have contributed to variations in compound efficacy, as has been previously noted (reviewed in reference 68). Differences in the results of the ATP metabolic assay and the MitoTracker Red CM-H_2XRos imaging-based viability assay are most likely due to not only those factors highlighted above but also the differences in starting gametocyte stage (mature stage V versus stages IV and V, respectively), duration of compound exposure (48 h of exposure versus 96 h of exposure, respectively), and the use of cryopreserved gametocytes in the ATP assay versus fresh gametocytes in the imaging-based assay. While the results for TSA and SAHA were generally consistent, the activity of CAY10603 was more variable. It may be that this compound has a steep dose-response curve that impacts activity at this concentration; however, concentrations higher than 5 μM were not examined in this study.

On the basis of the results of initial studies with the three commercial HDAC inhibitors, the MitoTracker Red CM-H_2XRos imaging-based viability assay was selected to further examine the activity of these and other HDAC inhibitors (10, 42). A panel of five structurally diverse HDAC inhibitors containing different cap groups and connecting-unit linker regions was assessed. As none of these compounds had previously been tested against asexual stage parasites, we first determined their asexual stage IC_{50}s against a drug-sensitive line (3D7) and a drug-resistant line (Dd2) of *P. falciparum*. While none of the compounds were as potent as TSA or SAHA, they all had selectivity for asexual stage parasites versus a normal mammalian cell line equivalent to or better than that of those two reference compounds. Some asexual stage structure-activity relationship data were apparent, with the alkoxyurea compounds 1 (IC_{50}, 0.25 μM) and 2 (IC_{50}, 0.45 μM) having more potent asexual stage activity than the alkoxysemicarbazide (compound 4; IC_{50}, 1.09 μM) and the amidoxime derivative (compound 5; IC_{50}, 1.42 μM). In contrast to data obtained for asexual stage parasites, none of the alkoxyurea compounds potently inhibited early- or late-stage gametocyte development (Table 1; IC_{50}s, 1.7 to >10 μM). Overall, the most potent gametocytocidal HDAC inhibitor was TSA, which had similar potency against both early- and late-stage gametocytes (Table 1; IC_{50}s, 0.09 and 0.07 μM, respectively) and better activity than the control compound, methylene blue. Generally, the selectivity of the HDAC inhibitors for gametocytes versus a normal mammalian cell was lower than that for asexual stages and similar to that of the reference compounds TSA and SAHA. These data demonstrate that while some HDAC inhibitors have promising gametocytocidal activity, raising the possibility of development of this class of compounds as new gametocytocidals in the future, improvements in both potency and gametocyte-specific selectivity are still required.

It is widely accepted that late-stage gametocyte activity equal to or better than that of asexual stage activity is essential for potential transmission-blocking drugs. Our data show that the *in vitro* gametocytocidal activities of the HDAC inhibitors examined in this study are generally less than their activities against asexual stage parasites (Table 1). Lower activity against gametocytes than asexual stages is not unique to our study (42), with possible explanations including variations in the expression of target proteins, alterations in compound uptake in the different life cycle stages, and the technical limitations of current assays. For example, recent *in vitro* work has shown that mature male gametocytes are more sensitive to some antimalarial compounds than female gametocytes (74). Given that gametocyte populations are dimorphic and female biased (~3 to 5 female gametocytes to 1 male gametocyte), future studies on this class of compounds will need to include assessments of sex-specific effects. An improved understanding of the target(s) of HDAC inhibitors in gametocytes may also help improve the profiles of these compounds, for example, through the targeting of particular HDAC isoforms. Other considerations that will need to be addressed include gametocyte viability effects versus developmental delay effects and the need for compounds that have appropriate pharmacokinetic profiles and compatibility with potential partner drugs.

In addition to killing early/late-stage gametocytes, downstream processes such as gamete formation in the mosquito may be targets of transmission-blocking agents. When we assessed the effect of the HDAC inhibitors SAHA and TSA on *in vitro* male gamete formation, we observed very low inhibitory activity (IC_{50}s, 200 to 500 μM). Although the exflagellation inhibition activity of compounds is generally markedly lower than that obtained against mature gametocytes (53, 74), the exflagellation IC_{50}s of SAHA and TSA were at least 600-fold higher than the late-stage gametocyte IC_{50}s (Table 1). Likewise, no activity against the *in vitro* conversion of *P. berghei* mouse malaria gametocytes to ookinetes was observed. Although we cannot preclude the possibility of more potent inhibitory activity by the use of longer exposure times, different classes of HDAC inhibitors, or inhibitors that target different isoforms in the parasite, our data indicate that the

hydroxamate-based HDAC inhibitors SAHA and TSA have very poor exflagellation and ookinete conversion inhibition activity. While transmission-blocking studies were not performed in this study, future work should also include transmission-blocking studies in mosquitoes infected with HDAC inhibitor-treated gametocytes.

Several HDAC inhibitors that are potent inhibitors of asexual blood-stage *P. falciparum* parasites, including SAHA and TSA, have been shown to inhibit recombinant *Pf*HDAC1 in *in vitro* enzyme assays (58). We and others have also demonstrated that hyperacetylation of parasite histone and nonhistone proteins and global transcriptional alterations are a functional consequence of HDAC inhibitor exposure in asexual stage parasites (31, 32, 58, 63, 69, 73, 75). This is consistent with a mode of action of this class of compounds that targets HDAC enzymes. When we examined the effect of three HDAC inhibitors with various gametocytocidal activities, TSA, SAHA, and CAY10603, on *P. falciparum* stage III and IV gametocytes, we observed increases in histone H3 and H4 acetylation profiles (Fig. 5). While these data provide evidence of a mode of action in targeting histone acetylation in gametocytes, it is not possible to directly correlate gametocytocidal activity with hyperacetylation data. To quantitate these changes, future studies will need to consider differences in the physiochemical properties of compounds (e.g., variations in log P [octanol-water partition coefficient]), compound potency, timing and speed of action, and variations in molecular targets, as well as profile the effect of these and other compounds on different acetylated histone forms (e.g., tetra-acetylated H4 versus monoacetylated forms). Unfortunately, many of the tools needed for such profiling are not currently available, in particular, recombinant forms of different *P. falciparum* HDAC proteins. Future work should also focus on determining whether the effect of HDAC inhibitors on gametocytes is due to direct targeting of HDAC action or indirect effects. Such profiling should include assessment of global transcriptional and acetylomic changes in treated versus untreated parasites. In contrast to the observed alteration to histone acetylation, nonhistone acetylation did not appear to be affected under the conditions utilized (see File S3 in the supplemental material). While this may mean that nonhistone protein acetylation is not a target of these HDAC inhibitors in gametocytes, additional work will be required to explore these apparent differences in more detail, including comparison of the effects on different developmental forms and on male versus female gametocytes and assessment of temporal responses and the effect of additional compounds.

The importance of lysine acetylation in *Plasmodium* growth and development has recently been highlighted by the identification of the first acetylome of *P. falciparum* parasites. A total of 421 acetylation sites in 230 proteins were identified in asexual stage parasites (59), with a significant divergence from those in other eukaryotes being noted. Not only were a large number of *Plasmodium*-specific proteins acetylated, but also different acetylation sites were identified in evolutionarily conserved acetylated proteins. This work, together with our findings, paves the way for similar acetylomic studies in *Plasmodium* gametocytes. Our work also identifies gametocytocidal HDAC inhibitors, such as TSA, to be potentially useful tools for dissecting the roles of HDACs in gametocyte development and on epigenetic regulation in this life cycle stage.

Our data show that HDAC inhibitors are a novel starting point for the development of new inhibitors of not only asexual (30) and exoerythrocytic (73) stage parasites but also gametocytes. Safe and effective antigametocyte drugs are crucial to any effort to eradicate malaria because of the persistence of gametocytes in patients who have been successfully treated for the disease. There are few known inhibitors of this life stage, and the only approved drug, primaquine, has significant safety concerns. Because HDAC inhibitors are also effective against asexual and exoerythrocytic parasite stages (30, 73), development of a suitable drug candidate for use in combination therapies to reduce gametocyte formation could avoid many of the ethical issues associated with gametocyte-specific drugs. HDAC inhibition, either directly or indirectly via targeting of essential coregulatory proteins unique to the *Plasmodium* parasite, thus presents a promising strategy for the development of novel antimalarial treatments.

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