Small-molecule histone methyltransferase inhibitors display rapid antimalarial activity against all blood stage forms in *Plasmodium falciparum*

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Edited by Thomas E. Wellems, National Institutes of Health, Bethesda, MD, and approved September 5, 2012 (received for review April 3, 2012)

Epigenetic factors such as histone methylation control the developmental progression of malaria parasites during the complex life cycle in the human host. We investigated *Plasmodium falciparum* histone lysine methyltransferases as a potential target class for the development of novel antimalarials. We synthesized a compound library based upon a known specific inhibitor (BIX-01294) of the human G9a histone methyltransferase. Two compounds, BIX-01294 and its derivative TM2-115, inhibited *P. falciparum* 3D7 parasites in culture with IC₅₀ values of ~100 nM, values at least 22-fold more potent than their apparent IC₅₀ toward two human cell lines and one mouse cell line. These compounds irreversibly arrested parasite growth at all stages of the intraerythrocytic life cycle. Decrease in parasite viability (>40%) was seen after a 3-h incubation with 1 μ M BIX-01294 and resulted in complete parasite killing after a 12-h incubation. Additionally, mice with patent *Plasmodium berghei* ANKA strain infection treated with a single dose (40 mg/kg) of TM2-115 had 18-fold reduced parasitemia the following day. Importantly, treatment of *P. falciparum* parasites in culture with BIX-01294 or TM2-115 resulted in significant reductions in histone H3K4me3 levels in a concentration-dependent and exposure time-dependent manner. Together, these results suggest that BIX-01294 and TM2-115 inhibit malaria parasite histone methyltransferases, resulting in rapid and irreversible parasite death. Our data position histone lysine methyltransferases as a previously unrecognized target class, and BIX-01294 as a promising lead compound, in a presently unexploited avenue for antimalarial drug discovery targeting multiple life-cycle stages.

chromatin | global health | chemical genetics

Malaria continues to claim >1 million lives annually, particularly in the vulnerable populations of pregnant women and children <5 y of age (1, 2). Although artemisinin-based combination therapies have helped rescue the world's antimalarial armamentarium, the parasite's ability to develop resistance continues to outpace our ability to control this devastating disease (3). Effective malaria drug discovery efforts must therefore include both the development of improved antimalarials from existing compounds and the discovery of new parasite drug targets and novel small-molecule inhibitors.

Epigenetic control of gene regulation in *Plasmodium falciparum*, the main causative agent of human malaria, has received considerable attention originally due to the apparent lack of recognizable transcription factors in the parasite genome (4). Although the recent discovery of a family of apicomplexan AP2 transcription factors (5, 6) has partly fulfilled the search for more traditional transcription factors, epigenetic gene regulation, particularly at the level of histone posttranslational modifications, has proven to play a significant role in *P. falciparum* virulence gene regulation. For example, expression of variant surface antigen gene families (7) and ligands involved in parasite red blood cell (RBC) invasion (8) are controlled by histone acetylation and methylation marks. Apicomplexan parasites, including *Plasmodium* and *Toxoplasma*, possess orthologs to many chromatin remodeling proteins and enzymes responsible for histone modifications (9–11), and many

conserved posttranslational modifications have been identified on *P. falciparum* histones (12). Genome-wide high-resolution ChIP-on-chip analysis revealed that the generally activating histone modifications trimethyl histone H3 lysine 4 (H3K4me3) and acetyl histone H3 lysine 9 (H3K9ac) are located throughout the parasite genome (13, 14). Whereas H3K9ac is associated with transcriptionally active genes throughout the erythrocytic life cycle, H3K4me3 appears to mark active and “poised” genes in blood-stage parasites. The generally repressive trimethyl histone H3 lysine 9 (H3K9me3) is only found associated with clonally variant gene families and telomeric regions, and it is apparently not involved in general transcriptional repression in *P. falciparum* as it is in other organisms (13). More specifically, the H3K9me3 and H3K4me2/3 histone marks are involved in the monoallelic expression of the *var* gene family (13, 15, 16), the most clinically relevant multicopy gene family, which encodes for PfEMP1, a protein trafficked to the infected erythrocyte surface that mediates cytoadhesion and contributes to immune evasion (17–19).

Histone methyltransferases are responsible for the addition of methyl groups to specific histone arginine or lysine residues. Aberrant histone methylation has been associated with a variety of human cancers, and as such protein methyltransferases are a current target class for the development for new cancer chemotherapies (20, 21). As for targeting parasite epigenetic gene regulation through histone posttranslational modifications, the few studies present in the literature have focused exclusively on modulating histone acetylation via the histone acetyltransferase (HAT) inhibitors curcumin (22) or anacardic acid (23) or the histone deacetylase (HDAC) inhibitors nicotinamide (24), apicidin (25), or derivatives of hydroxamic acid (26–28). Although the characterization of *P. falciparum* protein arginine *N*-methyltransferase (PRMT1) activity and inhibition has been described (29), there are no reports of drug discovery efforts targeting the parasite histone lysine methyltransferases (HKMTs). The *Plasmodium* genome contains at least four SET-domain-containing methyltransferases with predicted H3K4 specificity and one with predicted H3K9 activity (9, 11, 15). Sequence homology between the parasite enzyme catalytic SET domains and representative human homologs MLL (H3K4 specific) and G9a (H3K9 specific) varies from 11% to 53% (Table S1), with catalytic residues being well-conserved (Fig. S1).

We have pursued *P. falciparum* HKMTs as a potential target class for the development of novel antimalarials. We synthesized and assessed a small focused compound library based on a known

Author contributions: N.A.M., T.A.M., S.M., A.S., and M.J.F. designed research; N.A.M., T.A.M., and S.M. performed research; T.A.M. and M.J.F. contributed new reagents/analytic tools; N.A.M., S.M., A.S., and M.J.F. analyzed data; and N.A.M., A.S., and M.J.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1205414109/-DCSupplemental.

Fig. 1. Chemical structures of parent compound BIX-01294, active derivative TM2-115, and inactive derivative TM2-119.

specific inhibitor, BIX-01294 (Fig. 1) was discovered in a high-throughput screen and was shown to be an inhibitor of the HKMTs G9a/GLP (30). BIX-01294 has also been used successfully in stem cell modulation (31, 32), and subsequent medicinal chemistry studies have shown the potential of this scaffold in the discovery of compounds with increased potency, selectivity, and cellular permeability (33–38). In this work, we identified two compounds that target histone methylation in *P. falciparum*. These compounds arrested parasite growth at all stages of the intraerythrocytic life cycle. Parasite growth arrest in treated cultures was rapid and irreversible. Mice with patent *Plasmodium berghei* ANKA strain infection treated with a single dose (40 mg/kg) of TM2-115 (Fig. 1) showed 18-fold reduced parasitemia the following day and survived for 3 wk without succumbing to cerebral malaria. This finding positions HKMTs as a previously unrecognized target class in malaria parasites and BIX-01294 as a promising chemical start point with broad and rapid activity against the different stages of parasite development.

Results

Initial BIX-01294 Compound Assessment. Our initial screen of BIX-01294 and a synthesized focused library of derivatives at a concentration of 2 μ M against *P. falciparum* 3D7 strain growth and proliferation in culture showed an essentially all-or-none effect on parasite viability in a SYBR Green-based 3-d assay. We chose two parasite active compounds, BIX-01294 and its derivative TM2-115, and one inactive compound, the derivative TM2-119, for further study (Fig. 1). Follow-up analysis revealed IC_{50} values for parasite killing of ~ 75 – 100 nM in a 3-d assay, with the most efficacious compound being the parent compound BIX-01294 (Table 1). These data reveal BIX-01294, a known histone methyltransferase inhibitor, and at least one of its derivatives to be submicromolar, cell-permeant inhibitors of malaria parasite growth in culture.

To investigate the efficacy of BIX-01294 and TM2-115 against existing drug resistant *P. falciparum* strains, we obtained IC_{50} values for these two compounds and chloroquine in a 3-d assay against the chloroquine-sensitive 3D7 strain parasites and the drug-resistant W2, 7G8, and Dd2 strain parasites. The data show an increase in IC_{50} for chloroquine in the drug-resistant strains relative to the 3D7 strain, but no corresponding increase in IC_{50} for either BIX-01294 or TM2-115 in these drug-resistant strains (Table 1). These results indicate that the parasite-killing activity of these compounds is unaffected by the multidrug-resistant phenotype of existing resistant malaria parasite strains.

To assess the general toxicity of the compounds that exhibited parasite growth inhibition in culture, we treated two human cell lines, JEG-3 placental choriocarcinoma cells and human foreskin

Table 1. IC_{50} values of chloroquine, BIX-01294, and TM2-115 against malaria parasites

<i>P. falciparum</i> strain (resistance)	IC_{50} , nM		
	CQ	BIX-01294	TM2-115
3D7 (sensitive)	16 $\pm$ 4	75 $\pm$ 36	100 $\pm$ 39
W2 (CQ)	377 $\pm$ 8	52 $\pm$ 3	116 $\pm$ 18
7G8 (CQ and pyr)	123 $\pm$ 5	40 $\pm$ 3	49 $\pm$ 9
Dd2 (CQ and MQ and pyr)	114 $\pm$ 8	72 $\pm$ 29	65 $\pm$ 13

P. falciparum growth and proliferation inhibition for chloroquine-sensitive 3D7 or drug-resistant W2, 7G8, and Dd2 strain parasites was performed with the 3-d SYBR Green I growth assay. IC_{50} values are mean $\pm$ SEM of the fitted inhibition curves from two to nine experiments of duplicate samples. CQ, chloroquine; MQ, mefloquine; pyr, pyrimethamine.

fibroblasts (HFF), and one mouse embryonic fibroblast (MEF) cell line with the two parasite-active compounds described above. A 3-d incubation of parasite-active compounds with mammalian cell lines revealed IC_{50} values for mammalian cell viability that were at least 22-fold higher than for parasite growth and proliferation (Table 2). These newly identified parasite-active compounds therefore display selectivity for parasites over the mammalian cell lines tested, suggesting that increased specificity can be derived through further compound refinement.

In Vitro *P. falciparum* Parasite Killing in Culture. A major goal of antimalarial drug discovery is to achieve rapid parasite death acting at every stage of the parasite's life cycle. To this end, we exposed highly synchronized *P. falciparum* parasites to our test compounds for three distinct, consecutive 12-h periods during the ~ 48 -h intraerythrocytic life cycle (Fig. 2A). Synchronized parasites were incubated with 1 μ M test compound for 12 h, and then cultures were washed and placed back into fresh culture to examine progression through the present cell cycle (Fig. 2B and C) and viability after 4 d of potential recovery (Fig. 2D). Giemsa smears of treated cultures at 50 h postinvasion (hpi), when reinvasion should be nearly complete, showed a large number of viable ring-stage parasites and a few remaining segmented late-stage schizonts in the DMSO-treated or 1 μ M TM2-119-treated cultures (Fig. 2B). In contrast, cultures treated for 12 h with 1 μ M BIX-01294 or TM2-115 showed virtually no ring-stage parasites at 50 hpi regardless of the cell-cycle stage in which they were treated (Fig. 2B). These active compound-treated cultures contained apparently dead or dying parasites approximately corresponding to the size and morphology of the stage in which the parasites were treated. Cytometry analysis showed a clear population of reinvasion rings in all DMSO-treated or TM2-119-treated cultures at 50 hpi, but ~ 10 -fold fewer cells in the same ring-stage gate in the BIX-01294-treated or TM2-115-treated cultures (Fig. 2C and Fig. S2). Although ring-stage parasites were virtually

Table 2. Compound specificity for parasite vs. mammalian cell killing

Compound	<i>P. falciparum</i> 3D7 IC_{50} , μ M	Mammalian cells					
		JEG-3		HFF		MEF	
		IC_{50} , μ M	SI	IC_{50} , μ M	SI	IC_{50} , μ M	SI
BIX-01294	0.075 $\pm$ 0.036	3.3 $\pm$ 0.8	44x	6.1 $\pm$ 2.4	81x	11 $\pm$ 2	147x
TM2-115	0.100 $\pm$ 0.039	2.2 $\pm$ 0.9	22x	5.7 $\pm$ 2.1	57x	8.7 $\pm$ 2.1	87x
TM2-119	>2	na	na	na	na	na	na

P. falciparum killing was performed with the 3-d SYBR Green I growth assay, mammalian cell toxicity was determined with the 3-d CellTiterBlue assay. Parasite IC_{50} values are from the data in Table 1, Mammalian IC_{50} values are from duplicate measurements from one representative experiment. All IC_{50} values are the mean $\pm$ SEM of the fitted data. na, not analyzed; SI, selectivity index.

Fig. 2. Stage-dependent antimalarial activity. (A) Highly synchronized *P. falciparum* parasites were treated for 12 h with 1 μ M test compounds or DMSO for three consecutive periods of the intraerythrocytic life cycle. (B) Treated cultures were analyzed by Giemsa smear for reinvasion, indicating completion of the intraerythrocytic life cycle, at 50 hpi. (C) Reinvasion at 50 hpi was quantified by flow cytometry. (D) Treated parasites were washed, diluted, and allowed to grow for 4 d (approximately two cycles), and the resulting parasitemia was quantified. Data in C and D are the mean $\pm$ SEM of 50,000 RBCs from duplicate samples.

absent from Giemsa smears of BIX-01294-treated or TM2-115-treated 50 hpi cultures, it is possible that viable but dormant parasites remained. To address this possibility, culture samples were washed after the 12-h compound treatment, diluted, and placed into fresh culture for an additional 4 d of growth. The resulting parasitemia of these cultures showed no outgrowth of BIX-01294-treated or TM2-115-treated parasites, regardless of the cell-cycle stage in which they were treated (Fig. 2D). Cultures treated with DMSO or TM2-119 grew to a virtually identical parasitemia level. These results show that BIX-01294 and TM2-115 treatment results in irreversible parasite growth arrest at all stages of the intraerythrocytic cell cycle.

With the understanding that BIX-01294 and TM2-115 are active against all stages of the parasite erythrocytic life cycle, we obtained growth curves for asynchronous 3D7 parasites treated with various concentrations of test compounds to more fully explore the kinetics of parasite killing by these compounds (Fig. 3*A* and *B*). The data show that DMSO-treated or TM2-119-treated parasites clearly proliferated during the 24-h treatment period, as did parasites treated with 100 nM BIX-01294 or 100–300 nM TM2-115. In the presence of 300 nM or 1 μ M BIX-01294 or 1 μ M TM2-115, parasite proliferation appeared to arrest entirely after 12 h of treatment. The histone methyltransferase inhibitor BIX-01294 and derivative TM2-115 therefore exerted a rapid killing effect against mixed-stage parasites in culture, a highly desirable characteristic for a potential antimalarial lead compound.

To more fully investigate the long-term effects of compound exposure in a time- and dose-dependent manner, samples of parasites treated with various concentrations of test compounds for 3, 6, 12, or 24 h were washed and diluted into fresh culture.

The parasitemia of these washed and diluted cultures was measured 4 d after the end of treatment to assess the effect of short exposure to and subsequent removal of test compound on parasite growth (Fig. 3 C and D). The parasitemia values revealed that exposure to BIX-01294 or TM2-115 results in a dose-dependent decrease in parasite viability, evident after as little as 3 h of compound exposure (>40% inhibition) and subsequent removal. Treatment with BIX-01294 at a concentration of ≥ 300 nM or TM2-115 at a concentration of 1 μ M resulted in virtually no parasite proliferation after 24 h of exposure and subsequent compound removal. These data together show that short exposure to submicromolar concentrations of BIX-01294 is sufficient to produce irreversible parasite killing.

In Vivo Effect on *P. berghei* Parasites in Infected Mice. The putative *P. falciparum* histone H3K4 and H3K9 methyltransferases have clear orthologs in the rodent malaria parasite *P. berghei* (9), displaying 75–93% identity among their catalytic SET domains (Table S1). To examine whether compounds that showed activity against *P. falciparum* parasites in culture also have an effect on malaria parasites in vivo, we examined parasite clearing in an acute infection murine model. Five mice per treatment group were infected with 5×10^6 *P. berghei* ANKA strain parasites. ANKA strain parasites are highly virulent, normally causing death by cerebral malaria within 1 wk after injection. On day 3 postinfection, mice were injected i.p. with a single 40 mg/kg dose of BIX-01294, TM2-115, chloroquine, or a DMSO vehicle control. Parasitemia was followed by flow cytometry every day thereafter to assess onset of activity and recrudescence (Fig. 4). BIX-01294 and TM2-115 were able to decrease parasitemia by 2- and 18-

Fig. 5. Histone methylation levels in treated parasites. (A) Asynchronous *P. falciparum* parasites were grown in varying concentrations of BIX-01294 or 1 μ M TM2-115 or -119, and samples were harvested at various time points for immunoblotting for histone H3 methylation levels. (B) Several Western blots were quantified by densitometry, and relative H3K4me3 levels, normalized to histone H3 core levels, were calculated. Densitometry values are from the analysis of Western blots from two to eight separate experiments and are the mean $\pm$ SEM normalized to the DMSO-treated sample for each treatment period. * $P < 0.05$ vs. DMSO control.

efficacy against *P. berghei* for compounds with nearly identical efficacy against *P. falciparum* may be attributed to differences in the compound targets or their expression levels, or it may be due to differential compound metabolism in the mouse. Hydroxamate-based HDAC inhibitors have been shown to cure less virulent NK65 *P. berghei* parasite infection at lower doses by i.p. injection, but required multiple doses over several days beginning immediately after parasite infection (27). The HDAC inhibitor apicidin revealed similar activity in vivo, but without a total cure (25). In comparison with other epigenetic targeting studies reporting in vivo effects, our results suggest considerable promise in targeting parasite histone methyltransferases using BIX-01294 derivatives.

Our parasite killing results show potential compared with other epigenetic regulation targeting programs, namely against parasite HATs and HDACs. The HAT inhibitor curcumin displays an IC_{50} of $>20 \mu$ M against parasites in culture, but treatment results in an increase in reactive oxygen species in addition to histone hypoacetylation, so the specificity of a purely epigenetic effect is not clear (22). Indeed, curcumin is a highly promiscuous compound that has been reported to hit a large number of diverse targets outside the histone-modifying enzymes (45). The HAT inhibitor anacardic acid is also effective against parasites in culture, although with an IC_{50} of $>30 \mu$ M (23). Inhibition of parasite HDACs has provided more potent compounds against parasite growth and proliferation (26–28, 46). For example, parasite killing by apicidin is reported with an IC_{50} of 90 nM, and the hydroxamate trichostatin A has been shown to inhibit parasite growth with an IC_{50} of ~ 10 nM (47). Further refinement of the hydroxamates has produced increasingly efficacious compounds for parasite killing, with mixed consequences with regard to parasite specificity and in vivo activity (26–28). However, although HDAC inhibitors have progressed into clinical practice for the treatment of certain types of tumors, there are still significant questions over whether their strong antitumorogenic efficacy relates to a truly epigenetic effect—i.e., effect gene transcription although hyperacetylation of histones (21). It is likely that similar issues may arise as the study of HDAC inhibitors as antimalarials progresses. Furthermore, significant issues remain in the design of isoform-specific HDAC inhibitors of mammalian HDACs because of the highly conserved active site of these enzymes (21). This issue has the potential to lower the therapeutic index of any antimalarial HDAC inhibitors based on the common HDAC inhibitor pharmacophore. Therefore, although parasite HDACs have thus far represented a promising avenue for the

discovery of novel malaria therapeutics, we believe our results now place parasite histone methyltransferases in a similarly appealing position.

BIX-01294 was originally discovered in a screen for inhibitors of human G9a, a histone H3K9 monomethylase and dimethylase, and was found to be selective for mainly G9a and the closely related H3K9 methyltransferase GLP (30). BIX-01294 was shown to be noncompetitive for the methyl donor *S*-adenosylmethionine and instead competitive for the histone substrate (30, 37). In light of the high precedence of this class of compounds to serve as mammalian HKMT inhibitors (33–38), focused BIX-01294 derivatives were explored for the development of parasite-specific histone methyltransferase inhibitors in our study. Our discovery that BIX-01294-treated and TM2-115-treated *P. falciparum* parasites exhibit greatly reduced H3K4me3 levels in BIX-01294-treated parasites, while maintaining a distinct profile compared with human cell lines treated with BIX-01294, is highly supportive of our compounds acting as parasite histone methyltransferase inhibitors. In comparison, the original BIX-01294 discovery reported a 20–40% decrease in H3K9me2 levels in mammalian cells treated with 4.1 μ M BIX-01294, a dose 2.4-fold the IC_{50} for the G9a enzyme, for 2 d (30). One could attribute the shift in inhibition specificity profile to differences between the *P. falciparum* histone methyltransferase enzymes and their orthologs from previously studied organisms. Functional parasite methyltransferase enzymes assays are needed to confirm this hypothesis; however, so far our attempts to express functionally active HKMT domains (Fig. S1) have resulted only in very low enzymatic activity. There is evidence that this small-molecule scaffold can be tuned to effect HKMT specificity (33–38), in particular altering activity against HKMTs other than G9a. It is highly likely that focused medicinal chemistry efforts will enable further specificity of these compounds toward their parasite targets.

In conclusion, we have identified highly active HKMT inhibitory compounds that produce rapid and irreversible *P. falciparum* killing of developing parasites. Given the important role of histone lysine methylation in parasite differentiation and proliferation, our results predict that our compounds would target almost all life-cycle stages of malaria parasites, including transmission stages. Only the dormant liver stage of the human malaria parasite *Plasmodium vivax* does not apparently differentiate and proliferate. However, it is tempting to speculate that liver-stage dormancy may be controlled by similar repressive methylation marks that silence the *var* gene family (13, 16).

These results position this family of enzymes as a previously unrecognized target class in the development of antimalarial chemotherapies, with BIX-01294 and close analogs as highly useful start points for further compound development. Aided by the eventual production of functional recombinant methyltransferase enzymes, potent and specific inhibitors can be developed to both examine epigenetic gene regulation in the parasite and to add much-needed chemical entities to the antimalarial pipeline.

Materials and Methods

Chemical Synthesis. BIX-01294 and analogs were synthesized according to well-established procedures (33–38). See [SI Compound Characterization Data](#) for characterization data for BIX-01294, TM2-115, and -119.

Parasite Culture. *P. falciparum* 3D7, W2, 7G8, and Dd2 strain parasites were cultured at 2–5% hematocrit and $<8\%$ parasitemia in RPMI 1640 containing 0.5% albumax by using established methods (48). Highly synchronous cultures were obtained through gelatin floatation followed by sorbitol treatment 4 h later. Parasitemia was routinely quantified by flow cytometry of infected RBCs fixed in PBS containing 0.25% glutaraldehyde, washed in PBS containing 50 mM ammonium chloride, and stained with 2.5–5 μ M SYTO-16 (42). Parasite stage was determined by Giemsa-stained blood smears.

Proliferation Assays. Three-day parasite proliferation assays were performed in 96-well plates with a starting parasitemia of 1% at a hematocrit of 2% by using SYBR Green I (49). Mammalian cell toxicity was determined by using Promega CellTiterBlue. Stage-specific proliferation was examined by using highly synchronized parasites in 24-well plates with a starting parasitemia of

1% at a hematocrit of 2%. Parasites were treated for 12 h with a 1 μ M test compound for one of three sequential periods of the life cycle. After the treatment period, parasites were washed once with warm RPMI 1640 medium and placed back into culture without test compound for analysis of reinvasion after the completion of one parasite cell cycle. Additionally, washed parasites were diluted 1:40 to assess proliferation of treated parasites 4 d after treatment. Time- and dose-dependent compound effects used larger asynchronous cultures at a starting parasitemia of ~2% and a hematocrit of 5%. Bulk cultures were treated with test compounds, and samples were collected at various time points for Western blots, for immediate parasitemia quantification by flow cytometry, and for compound washout and dilution into fresh culture for subsequent parasite proliferation quantification.

P. berghei Experiments. Five CL57BL/6 mice per treatment group were infected with 5×10^6 *P. berghei* ANKA strain GFP-expressing parasites (50). Three days postinfection, mice were treated with a single 40 mg/kg i.p. injection of test compound or equivalent DMSO control. Parasitemia was quantified from blood samples collected every day thereafter by flow cytometry of 50,000 RBCs and confirmed by Giemsa-stained blood smears.

Western Blots. Treated parasites, routinely stored at -80°C as infected RBC pellets, were obtained from several rounds of RBC lysis in cold PBS containing 0.1% saponin and protease inhibitors. Free parasites were lysed in 2% (wt/vol) SDS by bath sonication. Parasite proteins were separated by SDS/PAGE on 12% Bio-Rad Criterion gels in Invitrogen Mes NuPAGE running buffer and transferred to nitrocellulose membranes by using an Invitrogen iBlot on program P3 for 6 min. Membranes were blocked in TBS-T-G (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20, 0.25% gelatin) and probed for histone H3 core (Abcam ab1792; 1:5,000), H3K4me3 (Abcam ab1012; 1:1,000), or H3K9me3 (Millipore 07-442; 1:1,000) 1° antibodies diluted in TBS-T-G, followed by anti-mouse HRP (GE NTA931) or anti-rabbit-HRP (GE NA-934) 2° antibodies diluted 1:5,000 in TBS-T-G, and then revealed by using Pierce SuperSignal West Pico or Femto chemiluminescent substrate. Densitometry analysis was performed by using ImageJ software (<http://rsbweb.nih.gov/ij/>).

ACKNOWLEDGMENTS. Cytometry was performed at the Institut Pasteur Imagopole. This work was supported by Bill and Melinda Gates Foundation Grand Challenge Exploration Award OPP1008567 and a European Research Council PLASMOESCAPE grant (A.S.). N.A.M. was supported in part by a Pasteur Foundation of New York fellowship.

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