Activity of Epigenetic Inhibitors against *Plasmodium falciparum* Asexual and Sexual Blood Stages

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**ABSTRACT** Earlier genetic and inhibitor studies showed that epigenetic regulation of gene expression is critical for malaria parasite survival in multiple life stages and a promising target for new antimalarials. We therefore evaluated the activity of 350 diverse epigenetic inhibitors against multiple stages of *Plasmodium falciparum*. We observed ≥90% inhibition at 10 μM for 28% of compounds against asexual blood stages and early gametocytes, of which a third retained ≥90% inhibition at 1 μM.

**KEYWORDS** malaria, *Plasmodium falciparum*, epigenetic inhibitors, small-molecule screen, antimalarial agents, drug screening

Despite substantial progress in reducing malaria infections and deaths over the past 2 decades, the disease remains among the greatest global health challenges, with 219 million cases and 435,000 deaths in 2017 (1). The emergence of drug and insecticide resistance now threatens to reverse these gains and highlights the need for new classes of antimalarials for use in combination therapies (2). To minimize the emergence and spread of resistance, these new antimalarials should have independent modes of action from existing therapies and be effective against multiple parasite stages, including the asexual blood stages responsible for the disease's clinical manifestation, and gametocytes, the sexual blood stages that mediate transmission. Recent studies have demonstrated the essential function of multiple genes involved in epigenetic regulation of gene expression in asexual blood stages (3–7). Many of these genes likely also play key roles during the substantial chromatin remodeling that occurs during the early stages of gametocytogenesis (8, 9).

Several epigenetic inhibitors have been approved for treatment of various cancers, with more actively being evaluated in clinical trials (10). Such inhibitors also hold promise as antimalarials, with several studies involving limited numbers of epigenetic inhibitors having found activity against one or more stages of malaria parasites (11–18). To evaluate the promise of targeting epigenetic processes more systematically, we decided to test known inhibitors of epigenetic targets to maximize the chance of finding compounds with potent antimalarial activity that would make promising starting points for identifying parasite targets and subsequent structure-activity relationship (SAR) studies to improve selectivity. To this end, we screened the two largest commercially available libraries of 209 (Selleckchem, Houston, TX) and 141 (Cayman Chemicals, Ann Arbor, MI) epigenetic inhibitors at 10 μM and 1 μM (see Fig. S1 and Data Set S1 in the supplemental material) against both asexual blood stages and gametocytes of *Plasmodium falciparum*. Using previously described assays, we determined activity against asexual blood stages (19), as well as early- and late-stage gametocytes (20) of the *P. falciparum* NF54 strain expressing the tandem dimeric tomato red fluorescent protein under the control of a *peg4* gametocyte promoter (21). For the 25 compounds included more than once, differing only by vendor or counterion, the mean response is reported, as responses to repeat compounds showed only
minimal variation (see Fig. S2), leaving 324 unique compounds across a broad range of epigenetic target classes (Table 1).

Of these, 150 exhibited greater than half-maximal activity at 10 μM against at least one stage (Fig. 1, Fig. S3, and Fig. S4; see Fig. S1 and Data Set S1 for activities of all compounds tested). Of all compounds, 45% (146) and 17% (54) had greater-than-half-maximal activity against asexual stages at 10 μM and 1 μM, respectively (Table 1, Fig. 1A, and Fig. S3A). Activity against early gametocyte stages was similar, with 37% (120) and 17% (55) of compounds exhibiting more than 50% inhibition at 10 μM and 1 μM, respectively. More than 90% inhibition was observed for 28% (92) of compounds against asexual blood stages at 10 μM, with 10% (32) retaining ≥90% activity even at 1 μM (Table 2). Against early gametocyte stages, 29% (93) and 10% (32) had 90% effective concentrations (EC90) below 10 μM and 1 μM, respectively. Despite differences in methods and parasite strains, our findings agree well with previous results for eight (panobinostat, belinostat, vorinostat, chaetocin, trichostatin A, BIX01294, CAY10603, and pracinostat) of these compounds that had been screened against either asexual stages or gametocytes (11–17).

Notably fewer compounds showed activity against mature gametocytes at 1 μM (Fig. S4 and S3B), possibly because the epigenetic changes that underlie sexual differentiation are initiated during earlier stages of gametocytogenesis (8, 9). Nevertheless, 13 compounds exhibited substantial activity against all three stages at 1 μM (Fig. 1B). Thirty-one of the most active compounds (EC90 < 1 μM) against asexual or early gametocyte stages were selected for more detailed dose-response studies (Fig. 1C and Fig. S3B). While the majority showed similar potency against asexual stages and early gametocyte stages, we found that 13 compounds exhibited a >2-fold difference in activity against these two stages (Fig. S5A).

When compounds were grouped based on reported epigenetic targets in higher eukaryotes, those effecting deacetylation, methylation, and phosphorylation of histones had hit rates between 35 and 40% at 10 μM for both asexual blood stages and gametocytes (Table 1). Indeed, histone deacetylase (HDAC) inhibitors have recently shown significant promise as multistage antimalarials (15, 22–25). Genome-wide mutagenesis studies in P. falciparum and the rodent malaria parasite Plasmodium berghei have indicated the essentiality of multiple genes encoding histone-modifying enzymes (6, 7). While phosphorylation of histone tails has been observed in P. falciparum blood stages (26), it remains unclear whether the observed activity of these kinase inhibitors is the result of diminished histone phosphorylation, as the kinases implicated in modification of histone tails in higher eukaryotes also perform other critical functions (see Fig. S3 for kinase inhibitor results).

Hit rates were lower for compounds targeting processes involved in demethylation, acetylation, binding of histone modifications (histone readers), and DNA methylation.

### Table 1: Epigenetic inhibitors with EC50 at 10 and 1 μM, grouped by reported epigenetic process targeted in higher eukaryotes

| Target class              | No. of compounds | 10 μM | 1 μM |
|---------------------------|-----------------|-------|------|
|                           | Asexual stages  | Stage I-II gametocytes | Stage I-II gametocytes | Stage IV-V gametocytes |
| Histone acetylation       | 10              | 1 (10) | 1 (10) | 0 (0) | 0 (0) |
| Histone deacetylation     | 85              | 43 (51) | 38 (45) | 25 (29) | 26 (31) | 7 (8) |
| Histone methylation       | 51              | 32 (63) | 24 (47) | 11 (22) | 12 (24) | 4 (8) |
| Histone demethylation     | 18              | 9 (50) | 7 (39) | 1 (6) | 2 (11) | 2 (11) |
| Histone phosphorylation   | 66              | 41 (62) | 38 (58) | 13 (20) | 13 (20) | 1 (2) |
| Histone PARPylation       | 22              | 5 (23) | 4 (18) | 0 (0) | 0 (0) | 0 (0) |
| Histone reader domains    | 28              | 6 (21) | 3 (11) | 0 (0) | 0 (0) | 0 (0) |
| DNA methylation           | 14              | 3 (21) | 1 (7) | 1 (7) | 1 (7) | 1 (7) |
| Other                     | 30              | 6 (20) | 4 (13) | 3 (10) | 1 (3) | 0 (0) |
| Total                     | 324             | 146 (45) | 120 (37) | 54 (17) | 55 (17) | 15 (5) |

nPercentage of active compounds (n = 2 or 3).
FIG 1 Epigenetic inhibitors with activity against *P. falciparum* blood stages. (A) Compounds with ≥50% inhibition against asexual or early gametocyte blood stages at 10 μM. A heat map of mean percent inhibition of asexual replication and early gametocyte maturation at 10 and 1 μM compared to (Continued on next page)
*P. falciparum* carries one or more genes involved in these pathways, and lower hit rates against these may indicate greater divergence from their mammalian homologs or nonessentiality of these pathways in blood stages. For example, all but three of the 28 inhibitors of histone readers target the recognition of acetylated histones by mammalian bromo-domains, which have been noted for their divergence in *P. falciparum* (5).

The HDAC inhibitor quisinostat was the most potent multistage active compound, as EC\textsubscript{50} values against all three stages were in the low-nanomolar range. Of the eight compounds more active against asexual stages, seven were HDAC inhibitors, while three histone methyltransferase (HMT) inhibitors, the DNA methyltransferase (DNMT) inhibitor SGI-1027, and the pan-Jumonji histone demethylase (HDM) inhibitor JIB-04 (27) were more effective against early gametocytes. Intriguingly, the DNA methyltransferase inhibitor SGI-1027 had EC\textsubscript{50}s in the low-nanomolar range against asexual and early gametocyte stages and was among active compounds against late gametocyte stages. Two recent papers also identified DNMT inhibitors as potent antimalarials (24, 28). Interestingly, the lone DNA methyltransferase homolog in malaria parasites was found to be dispensable for asexual growth in *P. falciparum* (6), suggesting a novel alternative target.

Sixty compounds with submicromolar EC\textsubscript{50}s were evaluated for toxicity against human HepG2 cells (HB-8065; ATCC) at 1 μM for 72 h using Cell-titer Glo assays (Promega), as previously described (29). Unsurprisingly, a majority of these compounds exhibited some toxicity at 1 μM (Fig. S6 and S3B), as compounds were included in these libraries based on activity against epigenetic processes in mammalian cells. Nevertheless, two histone methyltransferases inhibitors, UNC0631 and UNC0642, exhibited promising selectivity, with antimalarial activity in the low-nanomolar range but low toxicity even at 1 μM.

Overall, this large screen shows that inhibitors targeting diverse epigenetic processes can effectively block asexual replication and sexual development of *P. falciparum* blood stages, confirming and extending findings of earlier studies targeting individual epigenetic enzyme classes. Our findings identified several additional inhibitors with nanomolar multistage activity and encouraging selectivity that offer a promising basis for additional SAR studies to improve both potency and selectivity for possible use as a new class of antimalarials with targets orthogonal to existing therapies.

**FIG 1 Legend (Continued)**

solvent-treated controls (n = 2; see Table S1 for complete data) is shown. Compounds are grouped based on the reported epigenetic process affected in higher eukaryotes: histone deacetylation (HDAC), histone acetylation (HAT), histone methylation (HMT), histone demethylases (HDM), DNA methylation (DNMT), and “other.” Gray shading indicates values excluded due to significant hemolysis at 10 μM. (B) Inhibition at 1 μM compared between early gametocyte stages (x axis), late gametocyte stages (y axis), and asexual stages (symbol color). The box lists 13 multistage-active compounds, with target categories indicated by the color of the compound names. (C) Additional analysis of dose response for 31 compounds with submicromolar EC\textsubscript{50}s against asexual stages or early gametocyte stages (n = 2 or 3).
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.08 MB.

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