Chromobacterium spp. mediate their anti-Plasmodium activity through secretion of the histone deacetylase inhibitor romidepsin

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The Chromobacterium sp. Panama bacterium has in vivo and in vitro anti-Plasmodium properties. To assess the nature of the Chromobacterium-produced anti-Plasmodium factors, chemical partition was conducted by bioassay-guided fractionation where different fractions were assayed for activity against asexual stages of P. falciparum. The isolated compounds were further partitioned by reversed-phase FPLC followed by size-exclusion chromatography; high resolution UPLC and ESI/MS data were then collected and revealed that the most active fraction contained a cyclic depsipeptide, which was identified as romidepsin. A pure sample of this FDA-approved HDAC inhibitor allowed us to independently verify this finding, and establish that romidepsin also has potent effect against mosquito stages of the parasite’s life cycle. Genomic comparisons between C. sp. Panama and multiple species within the Chromobacterium genus further demonstrated a correlation between presence of the gene cluster responsible for romidepsin production and effective antiplasmodial activity. A romidepsin-null Chromobacterium spp. mutant loses its anti-Plasmodium properties by losing the ability to inhibit P. falciparum HDAC activity, and romidepsin is active against resistant parasites to commonly deployed antimalarials. This independent mode of action substantiates exploring a chromobacteria-based approach for malaria transmission-blocking.

In spite of remarkable progress toward its elimination throughout the last decade, malaria remains endemic in 91 countries, with nearly half of the world’s population at risk in 2016 (212,000,000 new cases and 429,000 deaths estimated in 2015)1. Containing the spread of malaria is mainly achieved by the deployment of bed nets and insecticide treatments. Poor compliance and resistance, however, hinder the effectiveness of these efforts. In parallel, antimalarial drugs have been instrumental in preventing the most aggressive and lethal forms of the disease caused by Plasmodium falciparum. However, due to the limited structural diversity within the chemical scaffolds of current clinically-available drugs and the increased incidence of drug resistance, new antimalarial compounds with novel modes of action must be identified2–4.

Bacteria of the genus Chromobacterium are Gram-negative β-proteobacteria of the Neisseriaceae family that occur as flagellated rods or cocci in water or soil environments5. Originally only comprised of C. violaceum – a purple-pigmented bacterium that has been associated with opportunistic infections in humans – the genus has been expanded over the past ten years and now comprises more than 8 fully-characterized species6–11. We have recently described the novel Chromobacterium sp. Panama, notable for inducing lethality in larvae and adult Aedes and Anopheles mosquitoes, as well as in vitro and in vivo antipathogenic activity against the malaria parasite and the dengue virus12. These properties render this bacterium an interesting candidate to control both mosquito populations and pathogen transmission, since manipulation of mosquito gut microbiota has proven successful through exposing mosquitoes to bacteria-spiked artificial nectars13.

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Concerning its anti-Plasmodium potential, C. sp. Panama was found to render Anopheles gambiae more resistant to malaria parasite infection when laboratory-reared mosquitoes were colonized by the bacterium prior to feeding on infectious blood. This anti-Plasmodium activity was proven to be mediated by bacteria-produced and secreted metabolites, as in vitro assays independent of the mosquito system showed potent activity against asexual and sexual (both gametocytes and ookinetes) stages of the parasite.

The goal of this study is to characterize the antiplasmodial activity of chromobacteria by isolating and characterizing the secreted factor responsible for Plasmodium inhibition. For that purpose, we combine in silico, in vitro and in vivo approaches to compare the anti-Plasmodium activity of a multitude of Chromobacterium species to conclude that romidepsin, a known histone deacetylase (HDAC) inhibitor, is responsible for the previously observed anti-Plasmodium activity. Romidepsin had already been shown to negatively impact P. falciparum asexual and sexual stages in vitro; here we further analyze the spectrum of this activity to include mosquito stages of the parasite and discuss potential applications of this discovery.

Results

Anti-Plasmodium activity of Chromobacterium sp. Panama fractionates with romidepsin. We have previously shown the in vitro inhibitory effect of the supernatant of C. sp. Panama cultures against different stages of the malaria parasite. To understand the nature of this antiplasmodial activity we employed a bioassay-guided fractionation approach by which the presence of active compounds against asexual stages of P. falciparum was evaluated following successive rounds of chemical partition and liquid chromatography of the C. sp. Panama supernatant. Mass spectrometric analysis was then used to identify compounds within fractions of interest.

First, the supernatant of a 72 h culture grown in LB medium at 30 °C was subjected to an n-butanol-based extraction. This chemical processing retained and concentrated the desired activity against asexual stages of P. falciparum. To further resolve the components of fraction F, orthogonal size-exclusion FPLC (100–7000 g/mol) was used. Collected fractions were dried, resuspended in 20% DMSO and assayed for antiplasmodial activity against P. falciparum asexual stages as before. Fraction F was found to contain the most anti-Plasmodium activity (Fig. 1C), and thus was carried forward for subsequent analysis.

Higher resolution UPLC separation of fraction F revealed, as expected, that the original crude fraction contained multiple components (Supplementary Fig. S1). Three major components as judged by UV absorption (UPLC) and total ion current (MS) were isolated and designated F-I, F-II and F-III. For F-I, and when sulfur was included as a potential element, the measured mass of 541.2151 gave a predicted elemental composition of C_{24}H_{37}N_{4}O_{6}S_{2}, with high confidence (Fig. 1D). Two prominent fragment ions were visible indicating sequential losses of m/z = 117 and 83, corresponding to fragment losses of –C_{2}H_{12}NO_{2} and –C_{2}H_{12}NO, respectively. The observed parent mass and the unusual predicted presence of two sulfur atoms led us to a tentative assignment of the structure to FR901228, or romidepsin, a metabolite previously described from C. sp. 968. This structure could be confirmed by unique features of the fragmentation pattern observed in its mass spectrum. Typically, peptide bonds cleave adjacent to the carbonyl to give stable acylium ion fragments. For romidepsin, however, an unusually favorable β-elimination releases the valyl peptide subunit with loss of a proton followed by normal peptide scission to render loss of this amino acid fully intact (Fig. 1D). Sequential loss of a dehydrobutyryl (Dbh, from dehydration of a Thr residue) unit was seen further in accord with the structure assignment to romidepsin.

While romidepsin has a mixed polyketide and non-ribosomal peptide (NRPS) biosynthetic origin, the principal natural products in F-II and F-III had significantly greater molecular weights (1357.6859 and 1195.6311, respectively), but proved to be identical NRPS products that differed only by the presence or absence of a glucose modification. Here the interplay of mass spectrometry, genome sequence information and the availability of in silico tools to predict the identity of common amino acid building blocks and their order in a NRPS product allowed us to propose a tentative hexapeptide substructure containing a specific site of N-methylation: H_{2}N-Thr–Tyr–Thr–Gln–Gly–N-Me-Thr–Xxx(Leu/His/Arg)-COOH. Other fragmentary genomic data pointed to another threonine-activating domain, a putative glycosyltransferase and the presence of a specialized loading domain associated with N-terminal acylation by long-chain β-hydroxyacids. High-resolution mass spectrometric observation of the higher mass product, F-II, gave a prominent m/z = 1195 fragment consistent with the loss of glucose, followed by a series of fragment ions mirrored precisely in the mass spectrum of F-III. These common fragments allowed the following residue sequence to be assigned: H_{2}N-Thr–Tyr–Dbh (from dehydration of Thr)–Gln–Gly–Thr–His–COOH in complete agreement with prediction (Supplementary Fig. S2). The exact masses of F-II and F-III (+glucose) and the unique signature of this shared hexapeptide substructure dictated with high probability that F-II and F-III correspond to the previously investigated antifungal agents Sch 20562 and 20561 (Supplementary Fig. S3).

To further resolve the components of fraction F, orthogonal size-exclusion FPLC (100–7000 g/mol) was employed and showed that this fraction was comprised of at least ten distinct entities as determined by UV absorbance (Fig. 1E). These were individually collected and assayed for antiplasmodial activity against P. falciparum asexual stages as before. Potent activity was observed in fractions F6 and F7 (Fig. 1F) and both subfractions gave mass spectrometric data fully consistent with the depsipeptide romidepsin. Why romidepsin elutes as two distinct peaks by size-exclusion FPLC is unclear; it is possible the macrobicyclic structure of romidepsin exists in two distinct structural conformations that are differentiated by a sizing resin, such as its oxidized and reduced...
forms. Fractions F2 and F3, on the other hand, returned mass signatures consistent with lipodepsipeptides Sch 20561 and Sch 20562; however, no significant antiplasmodial activity was detected from these compounds in our in vitro assay against asexual stages of Plasmodium (Fig. 1F).

**Figure 1.** Bioassay-guided fractionation of Chromobacterium sp. Panama culture supernatant points to romidepsin as the main antiplasmodial compound. (A) Variation in growth of asexual stage Plasmodium falciparum NF54 upon incubation with culture supernatant (LB, 72 h, 30 °C) of C. sp. Panama, Sup., when compared to that upon incubation with the respective n-butanol extract, BuOH and successive 1:2 dilutions. The equivalent of 5 mL of culture was used in each case. 0% inhibition is adjusted for parasite growth in vehicle control (1% DMSO) and 100% inhibition is matched to that of 250 nM chloroquine. Results are shown as mean ± standard deviation of three technical replicates per two biological replicates (total of 6 values); significance was determined using a one-tailed one sample t-test to determine whether each treatment significantly lowered parasite growth compared to control (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001). CQ, chloroquine. (B) Reverse-phase FPLC chromatogram (absorbance at 210 nm) of n-butanol extract of 250 mL of culture supernatant of C. sp. Panama (LB, 72 h, 30 °C). Fraction boundaries (A-F) indicated in red; fraction F highlighted. Column: RESOURCE RPC 3 mL. Flow rate: 2 mL/min. Gradient elution with 2%-85% methanol in water 0.1% TFA (dashed line). (C) Variation in growth of asexual stage P. falciparum NF54 upon incubation with fractions recovered from reverse-phase FPLC (cf. 1B). Fractions were dried and resuspended in proportional amounts of 20% DMSO according to their initial volume. Data presented as 1 A. FT, flow-through. (D) Total ion chromatogram from UPLC-ESI-MS analysis of fraction F6. Structures of romidepsin and previously characterized fragmentation products are shown. (E) Size exclusion FPLC chromatogram (absorbance at 210 nm) of fraction F (cf. 1B). Fraction boundaries (F1-F10) indicated in red; fractions F6 and F7 highlighted. Column: Superdex Peptide 10/300 GL. Flow rate: 1 mL/min. Isocratic elution with 20% DMSO in water. (F) Variation in growth of asexual stage P. falciparum NF54 upon incubation with fractions recovered from size-exclusion FPLC (cf. 1E). Fractions processed as 1C; data presented as 1A.

**Antiplasmodial Chromobacterium spp. are closely related genetically and encode for romidepsin production.** In parallel with our bioassay-guided fractionation efforts, and to better understand the scope and nature of the anti-Plasmodium activity of species belonging to the Chromobacterium genus, we obtained a variety of bacterial strains (Table 1) and evaluated their culture supernatants for inhibitory effects on asexual Plasmodium stages. Upon parallel n-butanol processing of supernatants from 72 h biofilm-forming cultures, our in vitro bioassays revealed that antiplasmodial activity was restricted to C. haemolyticum (MDA0585 and W10 strains) and C. sp. Panama (Fig. 2A). Other species induced a non-significant variation in the growth of Plasmodium parasites when compared to vehicle control (1% DMSO), indicating that under our culture conditions only certain species are able to successfully express and secrete the factors responsible for parasite inhibition.

Next, we sought to understand and differentiate the strains exerting anti-Plasmodium effects from those that do not. Having access to genomic information for all species assayed (Table 4), we employed genome average nucleotide identity (gANI) to measure similarities and distances across the entire genomes. An UPGMA tree of these results indicated that C. haemolyticum and C. sp. Panama are closely related and cluster away from the remaining chromobacteria tested (Fig. 2B). This close relationship is consistent with the observation that these two species, and these two alone, seem to carry significant antiplasmodial activity. The distinct clustering of these
Lack of evidence implicating homoserine lactones as antipathogenic agents led us to discard this cluster. 01 is predicted to encode a homoserine lactone, a known bacterial signaling molecule related to quorum sensing. A calculated IC50 of 150.7 nM (95% CI: 114.1–198.9 nM). This finding is in agreement with data generated comparing these experiments with pure romidepsin, allowing us to control the dose being administered. As shown in Fig. 3A, romidepsin alone exhibits significant activity against this stage of the Plasmodium life cycle, with a calculated IC50 of 150.7 nM (95% CI: 114.1–198.9 nM). This finding is in agreement with data generated against drug-sensitive and NF54-derived P. falciparum strain 3D7 having an asexual IC50 in the 90–140 nM range for this drug, which was reported in the context of screening multiple clinically-approved HDAC inhibitors for anti-parasitic activity[41,42]. Given its potency, we hypothesize that romidepsin is the main antimalarial effector of Chromobacterium spp., and is unlikely to require other chromobacteria-produced factors for its anti-Plasmodium activity.

Next, we investigated the transmission-blocking activity of romidepsin by treating P. falciparum NF54 gametocyte cultures with the drug prior to feeding to A. gambiae females and evaluating mosquito infection at the post-fertilization forms; pyrvinium pamoate, a known gametocytocidal compound 40, was used as positive control. Treatments with 400 and 1000 nM of romidepsin, but not 64 or 160 nM, led to significantly impaired parasite growth within the mosquito (Fig. 3B), which compares to the in vitro IC50 of 637 nM previously reported against sexual stages of the less-robust gametocyte-producing strain P. falciparum 3D7[16]. This indicates that romidepsin has activity against sexual stages of P. falciparum and thus can potentially negatively impact transmission.

### Table 1. Bacterial strains in this study.

| Species          | Strain         | Source            | Abbreviation | Reference |
|------------------|----------------|-------------------|--------------|-----------|
| C. aquaticum     | CC-SEYA-1      | DSMZ (DSM 19852)  | CAQU         |           |
| C. haemolyticum  | MDA0585        | DSMZ (DSM 19808)  | CHAE         | 8         |
| C. piscinae      | LMG 3947       | DSMZ (DSM 23278)  | CPSI         | 9         |
| C. pseudoviolaceum | LMG 3953   | DSMZ (DSM 23279)  | CPSE         |           |
| C. subtusae      | PRAA4-1        | DSMZ (DSM 17043)  | CSUB         | 6         |
| C. vaccini       | MWU205         | DSMZ (DSM 25150)  | CVAC         | 10        |
| C. violaceum     | ATCC 12472     | ATCC              | CVIO         |           |
| Chromobacterium sp. | 968          | Yi-Qiang Cheng   | C968W        | 17        |
| Chromobacterium sp. | 968 ΔdepA    | Yi-Qiang Cheng   | C968A        | 15        |
| Chromobacterium sp. | Panama       | CSPP              | C968        | 18        |
| Chromobacterium sp. | W10          | NRRL (B-11053)   | CW10         | 19        |

**Romidepsin has potent anti-Plasmodium activity.** Taken together, our bioassay-guided fractionation approach and the genomic analysis of active chromobacteria strongly suggests romidepsin as the source of the antimalarial properties of these species. Romidepsin, marketed as Istodax®, is a potent histone deacetylase (HDAC) inhibitor and an FDA approved cancer therapeutic against T-cell lymphoma[36–38]. To follow up on our initial findings, we obtained this compound from a commercial source and proceeded to comprehensively study its effects on asexual, sexual and mosquito stages of the parasite.

As our preliminary assays focused on growth inhibition of asexual P. falciparum NF54, we started by replicating these experiments with pure romidepsin, allowing us to control the dose being administered. As shown in Fig. 3A, romidepsin alone exhibits significant activity against this stage of the Plasmodium life cycle, with a calculated IC50 of 150.7 nM (95% CI: 114.1–198.9 nM). This finding is in agreement with data generated against drug-sensitive and NF54-derived P. falciparum strain 3D7 having an asexual IC50 in the 90–140 nM range for this drug, which was reported in the context of screening multiple clinically-approved HDAC inhibitors for anti-parasitic activity[41,42]. Given its potency, we hypothesize that romidepsin is the main antimalarial effector of Chromobacterium spp., and is unlikely to require other chromobacteria-produced factors for its anti-Plasmodium activity.

Next, we investigated the transmission-blocking activity of romidepsin by treating P. falciparum NF54 gametocyte cultures with the drug prior to feeding to A. gambiae females and evaluating mosquito infection at the oocyst level. Gametocytes were washed ahead of feeding to remove traces of romidepsin, and ensure the observed effects were due to the drug's activity against early Plasmodium sexual stages and not against gametes or other post-fertilization forms; pyrvinium pamoate, a known gametocytocidal compound[39], was used as positive control. Treatments with 400 and 1000 nM of romidepsin, but not 64 or 160 nM, led to significantly impaired parasite growth within the mosquito (Fig. 3B), which compares to the in vitro IC50 of 637 nM previously reported against sexual stages of the less-robust gametocyte-producing strain P. falciparum 3D7[16]. This indicates that romidepsin has activity against sexual stages of P. falciparum and thus can potentially negatively impact transmission.
Our initial findings of antiplasmodial activity of *C. sp. Panama* were seen against mosquito stages of *P. falciparum*. To understand if romidepsin has activity against stages preceding the oocyst in *An. gambiae*, mosquitoes were fed increasing concentrations of romidepsin (vehicle: 0.5% DMSO, 3% sucrose) for 24 h prior to ingestion of a *P. falciparum* NF54 gametocyte-containing blood meal. After 7–8 days post infection, mosquitoes were dissected, and the number of oocysts in each midgut was counted (Fig. 3C). Mosquitoes that were allowed to feed on a 50, 200 or 1,000 µM romidepsin solution were significantly less infected than the control. While these concentrations are greater than the IC50 observed in vitro, in a 24 h period the mosquitoes will only ingest microliters of the solution, rendering the effective concentration of romidepsin available upon *Plasmodium* infection far lower than that of the original source. Observed variations in infection levels can be explained by variations in the amount of romidepsin ingested by the mosquitoes pre-infection. Furthermore, temporary lethargy was observed in mosquitoes fed at the highest concentrations of the drug, explaining why some failed to complete ingestion of the infected blood meal being, therefore, censored. Mosquito survival upon feeding on the different romidepsin-containing solutions was not affected when compared to control (Supplementary Fig. S4).

Romidepsin production is required for *Chromobacterium spp.* anti-*Plasmodium* properties. Having established that chromobacteria with antiplasmodial properties produce romidepsin, and having demonstrated the ability of romidepsin to inhibit growth and maturation of *P. falciparum* in vitro and in vivo, it became essential to understand if romidepsin production was a necessary and sufficient condition for the anti-*Plasmodium* effect seen in supernatants of *Chromobacterium spp.* cultures. For this purpose, n-butanol extracts of *C. sp. 968* and a derived mutant lacking the *depA* gene and thus incapable of secreting romidepsin were tested against asexual stages of *P. falciparum* as before. The ∆*depA* mutant was found to have no antiplasmodial activity in vitro (Fig. 4A).

The romidepsin-null mutant was also fed to *An. gambiae* mosquitoes and its induced lethality did not differ from that of wildtype control (Supplementary Fig. S5), indicating that romidepsin does not appear to be significantly contributing to the mosquitocidal activity previously seen with *C. sp. Panama* against adult *Aedes* and
While crete speciation assignment at this point. The absence of a full genomic sequence for the 968 strain precludes us from making a con-

C. sp. 968 not being a strain and belonging to a separate cluster of chromobacteria able to C. violaceum point to

Furthermore, tion of a branch of chromobacteria around C. haemolyticum that exclusively possesses antiplasmodial activity.

HDAC activity and romidepsin is active against Chromobacterium spp P. falciparum. inhibit

Anopheles mosquitoes. Upon infection with P. falciparum NF54, mosquitoes that had previously fed on the wild-type or ∆depA bacteria exhibited different infection levels: Plasmodium maturation was significantly impaired in those that ingested wildtype C. sp. 968, whereas those exposed to the romidepsin-null mutant showed no varia-

tion compared to the control (Fig. 4B). Of note, the reduction in the number of mosquitoes between the control those that ingested wildtype

Table 2. Nucleotide alignment results (megabLAST) querying putative biosynthetic gene clusters detected in the Chromobacterium sp. Panama genome by antiSMASH against the genomes of the remaining Chromobacterium spp. used in this study. Similarity index compounded by multiplying query cover with % identity; full results available in Supplementary Table S1. A similarity index of 90% or above indicates that C. sp. Panama gene cluster is present in the genome of that particular species; one of 30% or below indicates with confidence that the cluster is not replicated in the other genome.

| Cluster | Type | Source | Size (kb) | megaBLAST similarity index |
|---------|------|--------|----------|----------------------------|
|         |      |        |          | CAQU | CHAE | CPIS | CPSE | CSUB | CVAC | CVIO | CW10 |
| Cluster 01 | Homoserine Lactone | antiSMASH | 20.7 | 20% | 89% | 9% | 23% | 21% | 25% | 23% | 90% |
| Cluster 02 | Saccharide | ClusterFinder | 25.0 | 65% | 86% | 66% | 66% | 65% | 60% | 70% | 86% |
| Cluster 03 | NRPS | antiSMASH | 73.0 | 28% | 86% | 25% | 28% | 28% | 28% | 28% | 86% |
| Cluster 04 | Fatty Acid | ClusterFinder | 33.7 | 68% | 91% | 60% | 65% | 69% | 66% | 65% | 91% |
| Cluster 05 | NRPS | antiSMASH | 45.1 | 27% | 91% | 24% | 28% | 27% | 28% | 28% | 90% |
| Cluster 06 | Terpene | antiSMASH | 21.7 | 31% | 93% | 31% | 27% | 31% | 24% | 27% | 84% |
| Cluster 07 | NRPS | antiSMASH | 43.2 | 23% | 47% | 23% | 26% | 23% | 24% | 25% | 52% |
| Cluster 08 | Putative | ClusterFinder | 15.3 | 49% | 83% | 37% | 37% | 50% | 54% | 57% | 87% |
| Cluster 09 | Putative | ClusterFinder | 4.4 | 52% | 88% | 54% | 39% | 51% | 54% | 39% | 80% |
| Cluster 10 | Putative | ClusterFinder | 9.5 | 71% | 94% | 71% | 72% | 72% | 72% | 94% |
| Cluster 11 | Polyunsaturated Fatty Acid/Other Ketosynthase | antiSMASH | 51.3 | 16% | 89% | 16% | 13% | 16% | 14% | 12% | 64% |
| Cluster 12 | Putative | ClusterFinder | 8.8 | 39% | 93% | 43% | 40% | 39% | 43% | 43% | 92% |
| Cluster 13 | NRPS | antiSMASH | 51.5 | 21% | 47% | 19% | 27% | 23% | 23% | 26% | 83% |
| Cluster 14 | Putative | ClusterFinder | 7.2 | 53% | 84% | 52% | 53% | 53% | 55% | 54% | 84% |
| Cluster 15 | Bacteriocin | antiSMASH | 10.8 | 28% | 42% | 28% | 28% | 28% | 28% | 33% | 72% |
| Cluster 16 | Putative | ClusterFinder | 8.5 | 6% | 85% | 0% | 3% | 3% | 5% | 3% | 81% |
| Cluster 17 | Bacteriocin | antiSMASH | 10.8 | 49% | 91% | 45% | 61% | 49% | 60% | 61% | 93% |
| Cluster 18 | NRPS | antiSMASH | 43.9 | 34% | 86% | 33% | 27% | 34% | 29% | 30% | 83% |
| Cluster 19 | Fatty Acid | ClusterFinder | 21.1 | 74% | 92% | 75% | 76% | 74% | 75% | 76% | 92% |
| Cluster 20 | Putative | ClusterFinder | 8.9 | 9% | 95% | 9% | 65% | 9% | 65% | 65% | 95% |
| Cluster 21 | Saccharide/NRPS | ClusterFinder | 81.2 | 40% | 60% | 27% | 36% | 38% | 31% | 34% | 70% |
| Cluster 22 | Saccharide | ClusterFinder | 30.1 | 30% | 33% | 28% | 29% | 30% | 31% | 28% | 33% |
| Cluster 23 | Putative | ClusterFinder | 6.5 | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 87% |
| Cluster 24 | Putative | ClusterFinder | 4.6 | 84% | 91% | 83% | 80% | 84% | 83% | 81% | 91% |
| Cluster 25 | Putative | ClusterFinder | 4.8 | 74% | 89% | 66% | 78% | 74% | 74% | 79% | 91% |
| Cluster 26 | NRPS | antiSMASH | 49.6 | 16% | 63% | 18% | 17% | 16% | 13% | 13% | 60% |
| Cluster 27 | Putative | ClusterFinder | 8.5 | 20% | 89% | 20% | 20% | 20% | 22% | 20% | 89% |
| Cluster 28 | Putative | ClusterFinder | 11.7 | 13% | 22% | 0% | 6% | 6% | 13% | 6% | 21% |
| Cluster 29 | Saccharide | ClusterFinder | 31.2 | 30% | 90% | 26% | 32% | 30% | 26% | 30% | 91% |
| Cluster 30 | NRPS | antiSMASH | 6.1 | 0% | 4% | 0% | 2% | 0% | 2% | 0% | 92% |
| Cluster 31 | NRPS | antiSMASH | 3.1 | 5% | 90% | 0% | 5% | 0% | 0% | 89% |

Anopheles mosquitoes. Upon infection with P. falciparum NF54, mosquitoes that had previously fed on the wild-
type or ∆depA bacteria exhibited different infection levels: Plasmodium maturation was significantly impaired in those that ingested wildtype C. sp. 968, whereas those exposed to the romidepsin-null mutant showed no varia-
tion compared to the control (Fig. 4B). Of note, the reduction in the number of mosquitoes between the control and the experimental groups is a result of the entomopathogenic properties of these chromobacteria.

These results clearly place C. sp. 968 among the Chromobacterium species with anti-Plasmodium activity. While C. sp. 968 was previously believed to be a strain of the C. violaceum species, upon phylogenetic analysis based on 16S rRNA gene sequence comparisons it became apparent that this strain clusters with C. haemolyticum and C. sp. Panama and not with C. violaceum (Fig. 4C). This observation is in agreement with our initial descrip-
tion of a branch of chromobacteria around C. haemolyticum that exclusively possesses antiplasmodial activity. Furthermore, C. sp. 968 colonies are tan, similar to those of C. haemolyticum and C. sp. Panama, and not purple like C. violaceum and other closely related species due to violacein production. These findings and observations point to C. sp. 968 not being a C. violaceum strain and belonging to a separate cluster of chromobacteria able to produce romidepsin. The absence of a full genomic sequence for the 968 strain precludes us from making a con-
crete speciation assignment at this point.

Chromobacterium spp. inhibit P. falciparum HDAC activity and romidepsin is active against drug resistant isolates. Romidepsin has been described as an HDAC inhibitor with relevant activity against a P. falciparum 3D7 nuclear extract. To test this in our system, we incubated a nuclear protein extract of asexual P. falciparum NF54 with an acetylated histone substrate in the presence of the drug. The ratio of
Figure 3. Romidepsin strongly inhibits Plasmodium. (A) Dose-response curve of romidepsin against *P. falciparum* NF54. 0% inhibition is adjusted for parasite growth in vehicle control (1% DMSO) and 100% inhibition is matched to that of 250 nM chloroquine. IC$_{50}$ is indicated together with the 95% confidence interval returned after fitting a curve to the normalized data using the least squares method with variable slope. (B) *P. falciparum* NF54 gametocyte cultures were treated with increasing concentrations of romidepsin, 1 µM pyrvinium pamoate (positive control) or vehicle alone (0.5% DMSO), washed, and fed to *A. gambiae* females. (C) Female *An. gambiae* were allowed to feed on romidepsin at the indicated concentrations in 0.5% DMSO 3% sucrose for 1 day prior to being given a *P. falciparum* NF54-infected blood meal; control group was fed on vehicle alone. Oocyst numbers determined 7 days later for two (B) and three (C) independent replicates are shown. Horizontal bars represent the median number of oocysts per treatment. Significance was determined using the Kruskal-Wallis test by comparing treatment groups to the control and correcting for multiple comparisons by the Dunn's test (ns, not significant; ***p < 0.001; ****p < 0.0001).

Figure 4. Romidepsin production is necessary and sufficient for the antiplasmodial activity of *Chromobacterium* spp. (A) Variation in growth of asexual stage *P. falciparum* NF54 upon incubation with $n$-butanol extracts of approximately 5 mL of culture supernatants (LB, 72 h, 30 °C) of *C. sp. 968* wildtype (wt) and depA-null mutant (ΔdepA). 0% inhibition is adjusted for parasite growth in vehicle control (1% DMSO) and 100% inhibition is matched to that of 250 nM chloroquine. Results are shown as mean ± standard deviation; significance was determined using a one-tailed one sample t-test to determine whether each treatment significantly lowered parasite growth compared to control (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001). CQ, chloroquine. (B) Female *An. gambiae* were provided either PBS, *C. sp. 968* wildtype (wt) or its ΔdepA mutant suspended within 3% sucrose. After 1 day of being allowed to feed on these suspensions, they were given a *P. falciparum* NF54-infected blood meal, and oocyst numbers were determined 7 days later; oocyst counts for three independent replicates are shown. Horizontal bars represent the median number of oocysts per treatment; inhibition (%) was estimated based on the comparison of these values to that of the PBS control. Prevalence represents the proportion of infected mosquitoes per group. Significance was determined using the Kruskal-Wallis test by comparing treatment groups to the control and correcting for multiple comparisons by the Dunn's test (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001). (C) Evolutionary relationships between *C. sp. 968*, other chromobacteria and other related species (*Neisseria gonorrhoeae* NCTC13800; *Neisseria meningitidis* LNP21362; *Pseudomonas aeruginosa* DSM 50071) based on 16S rRNA genomic sequences as inferred by the UPGMA method. The optimal tree with the sum of branch lengths equal to 0.33018119 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis was conducted in MEGA6 and involved 13 nucleotide sequences for a total of 925 positions in the final dataset.
remaining acetylated histone substrate in romidepsin-treated vs. no-drug control was determined by ELISA after 1h incubation. Romidepsin suppressed the HDAC activity of the *P. falciparum* nuclear protein extract in a concentration-dependent manner (from 21% inhibition at 1 nM to 80–84% at 1–100 µM; Fig. 5A). Romidepsin has been characterized as a potent inhibitor of human class I HDAC enzymes and a weak inhibitor of class II HDACs, while there is no known activity against class III enzymes. *P. falciparum*, in turn, expresses at least 5 HDAC enzymes: one homologue of class I (PfHDAC1), two of class II (PfHDAC2 and PfHDAC3) and two of class III (PfSir2A and PfSir2B). A maximum inhibition of ~80% of *P. falciparum* HDAC activity by romidepsin even at high concentrations could be explained by its lack of activity against the class III enzymes together with a
Potential partial inhibition of the class II HDACs; specific activity against the class I member PfHDAC1 has been previously demonstrated\(^4\).

Given the results presented thus far, it is expected that a romidepsin-positive culture supernatant extract of wildtype *C. sp.* 968 possesses inhibitory activity against *Plasmodium* HDACs, the contrary being true for a romidepsin-negative extract of its \(\Delta\)depA mutant. To test this hypothesis, \(n\)-butanol extracts of comparable culture supernatants of these bacterial strains were prepared as before and tested for anti-HDAC activity via ELISA as above. Inhibitory HDAC activity was detected for the wildtype strain extract and its dilutions (from 57% inhibition at 1:25 to 80% undiluted, Fig. 5B), but not for the \(\Delta\)depA extract. This finding further provides a conclusive link between romidepsin-production and anti-*Plasmodium* activity of *Chromobacterium spp.*, now substantiated at a mechanistic level.

We then tested the inhibitory activity of romidepsin against *P. falciparum* strains known for being resistant to commonly used antimalarial drugs to understand if romidepsin is able to bypass their resistance mechanisms. These strains included *P. falciparum* CamWT_C580Y, a K13-propeller mutant associated with increased resistance to artemisinin; Dd2, resistant to chloroquine, pyrimethamine and mefloquine; GB4, resistant to chloroquine; and SB1-A6, resistant to inhibitors of cytochrome bc1 electron transport such as atovaquone\(^4\). Compared to *P. falciparum* NF54, which exhibited an IC\(_{50}\) of 150.7 nM (95% CI: 114.1–198.9 nM, Fig. 3A), strains CamWT_C580Y (IC\(_{50}\) = 190.1 nM, 95% CI: 114.9–249.6 nM), Dd2 (IC\(_{50}\) = 191.2 nM, 95% CI: 156.5–233.7 nM) and GB4 (IC\(_{50}\) = 156.9 nM, 95% CI: 120.1–205.0 nM) all showed overlapping intervals for IC\(_{50}\) values with 95% confidence (Fig. 5C–E), indicating no significant difference in activity of romidepsin against these drug-resistant variants compared to wildtype. An IC\(_{50}\) of 130 ± 40 nM previously reported for romidepsin against *P. falciparum* Dd2\(^4\) is comparable to our data. Therefore, phenotypic resistance to artemisinin, chloroquine, pyrimethamine and mefloquine does not appear to alter the inhibitory activity of romidepsin towards *P. falciparum*. For *P. falciparum* SB1-A6, the calculated IC\(_{50}\) was 228.8 nM (95% CI: 214.5–244.1, Fig. 5F), representing a 1.5 fold increase when compared to that of the NF54 strain. While this was a significant change, the low resistance factor of 1.5 – or 1.08 when taking the closest values in the boundaries of each of the confidence intervals – seems rather negligible and does not provide any strong evidence that the anti-*Plasmodium* activity is hindered when the parasite lacks requirement for electron transport through the cytochrome bc1 complex.

**Discussion**

We have previously established that *C. sp.* Panama is able to limit the development of the malaria parasite through colonization of the *An. gambiae* midgut, and that its bacteria-free culture supernatant inhibits *Plasmodium* growth in *vitro*\(^2\). In the present work, we further probe into this anti-*Plasmodium* activity, showing by bioassay-guided fractionation and multigenomic comparative analysis that romidepsin is the most likely *Chromobacterium*-produced metabolite responsible for its antiplasmodial activity. Using romidepsin obtained from a commercial source, we demonstrate its potent inhibitory effect against asexual, sexual and mosquito stages of the parasite’s life cycle. Furthermore, we validate that a romidepsin-null *Chromobacterium* mutant loses its anti-*Plasmodium* effect both against blood and mosquito stages. As comparable amounts of drug are needed to inhibit resistant variants of *P. falciparum* to commonly deployed antimalarial drugs, romidepsin seems to exert its activity by a distinct mechanism; ours and others’ *in vitro* measurements show its ability to limit HDAC activity of *P. falciparum* nuclear protein extracts, pointing to its mode of action as an HDAC inhibitor against these parasite enzymes crucial for regulation of gene expression.

Romidepsin was first characterized as an inhibitor of human class I and class II HDAC enzymes and is currently FDA-approved for treatment of T cell lymphoma\(^6\). Our data indicate that this drug is also potentially effective as an antimalarial with an IC\(_{50}\) of 150.7 nM against asexual *P. falciparum* NF54. This finding is in agreement with data generated by others against drug-sensitive *P. falciparum* 3D7\(^14,15\). For gametocytocidal activity, our data are comparable to that reported also in *P. falciparum* 3D7 in the context of screening for repurposing multiple approved drugs for malaria control\(^8\). Previous observations showing that treatments with romidepsin led to hyperacetylation of both histone and non-histone *P. falciparum* proteins, and relative inhibition of recombinant PHDAC1\(^4\), further reinforce the conclusion that it is the HDAC inhibitory activity of romidepsin that likely underlies its anti-*Plasmodium* effects. In fact, HDAC inhibitors have been pursued as antiparasitic drugs since the discovery in the mid-1990s that the also cyclic tetrapeptide apicidin targets *Plasmodium* and other Apicomplexa by limiting HDAC activity\(^9\). FR23522, yet another natural cyclic tetrapeptide, has also been described for its anti-apicomplexan properties by targeting TgHDAC3 in *Toxoplasma gondii*\(^6\), a class I HDAC with homology to PHDAC1 that shares an Apicomplexa-specific two-residue insertion within the catalytic site of the enzyme\(^7\).

| Strain          | Source: BEI Resources | Resistance traits                        | IC\(_{50}\)/nM |
|-----------------|-----------------------|------------------------------------------|---------------|
| CamWT_C580Y    | MRA-1251              | artemisinin                              | ND            |
| Dd2            | MRA-150               | chloroquine, pyrimethamine, mefloquine   | 87.8 [41.6–185.5] (chloroquine) |
| GB4            | MRA-925               | chloroquine                              | \(\geq12,500\) (chloroquine) |
| NF54           | MRA-1000              | drug sensitive                           | 7.8 [1.8–34.4] (chloroquine) |
| SB1-A6         | MRA-1002              | cytochrome bc1 inhibitors (*e.g.* atovaquone)\(^4\) | 4065 [888–18613] (atovaquone) |

**Table 3.** *Plasmodium falciparum* strains in this study. IC\(_{50}\) for a representative drug is indicated together with the 95% confidence interval returned after fitting a curve to the normalized data using the least squares method with variable slope.
As is true for these other cyclic tetrapeptides, however, romidepsin does not appear to be an ideal candidate as indicated by others electing to decline performing further tests with this drug in view of its selectivity index. The ratio between effective inhibitory dosages against mammalian and Plasmodium targets indicates that there would be an unacceptable level of side-effects if this drug were to be used in its current form. The majority of patients experience nausea, vomiting and anorexia, and some progressive fatigue and occasional fever, when undergoing romidepsin regimens. There is, nonetheless, some promise in considering romidepsin as a lead compound for studies to develop a novel drug with increased selectivity for Plasmodium HDAC enzymes when compared to their human counterparts, as has been explored with some success for apicidin.

While the direct use of romidepsin as a therapeutic drug against malaria cannot be advocated at this time, the same cannot be said when it comes to its transmission-blocking capabilities. Faithful to our original approach of using Chromobacterium spp. to suppress parasite infection of the mosquito vector, uncovering romidepsin as the causal agent of its anti-Plasmodium effect reinforces the value of this strategy. Control of mosquito populations remains the most widespread and perhaps most valuable strategy for malaria control. As discussed, chromobacteria are able to exert entomopathogenic activity in the malaria vector An. gambiae when colonizing their mid-gut through a mechanism that appears to be independent of their ability to secrete romidepsin (Supplementary Fig. S5). For those mosquitoes that survive this colonization, however, the mechanisms for a second-line control level are now substantiated. Chromobacterium spp. will secrete romidepsin and suppress parasite infection in the mosquito, leading to a potential transmission-blocking that would have an epidemiologically significant impact. This compound is shown for the first time to have a significant limiting effect on mosquito stages of the Plasmodium life cycle, and it does so by a mechanism distinct from those of currently deployed antimalarials. As such, it is not expected that any pressure applied on the parasite by this HDAC inhibitor as it cycles through the mosquito will result in resistance to any existing antimalarial drug, placing the use of a chromobacteria-based strategy against anopheline mosquitoes as an important additional tool for an integrated approach to malaria control. Further studies currently underway in the semi-field with Chromobacterium-spiked attractive sugar baits will determine the viability of this approach.

**Methods**

**Ethics Statement.** Anonymous commercial human blood (Interstate Blood Bank Inc.) was used for parasite cultures and mosquito feeding, and informed consent was therefore not applicable. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

**Bacterial cultures and n-butanol extraction.** Unless otherwise noted, Chromobacterium spp. (Table 1) were grown for 72 h at 30 °C in LB Lennox broth (Sigma, L3022) without agitation, allowing for the formation of a biofilm at the surface of the culture. For n-butanol-based extraction, cultures were then thoroughly mixed 1:1 with H₂O-saturated n-butanol and the top organic phase was recovered. Following a short evaporation step under reduced pressure, the mixture was run through a filter (particle retention size of 10 μm) and the filtrate was then evaporated in its entirety and the resulting residue resuspended in methanol. Subsequently, the sample was added to the same volume of 1:1 petroleum ether/diethyl ether under constant agitation, filtered (particle retention size of 1 μm) and the residue obtained resuspended in DMSO and stored at −20 °C until further use. To ensure comparability, cultures of different chromobacteria were run in parallel and equivalent volumes of culture and solvents applied to the chemical extraction protocol.

**Plasmodium falciparum strains and cultivation.** P. falciparum strains (Table 3) were maintained in continuous culture according to the method described by Trager and Jensen. Briefly, P. falciparum was grown in O + red blood cells at 2% hematocrit and RPMI 1640 medium supplemented with 10 mM glutamine, 25 mM HEPES, 50 μg/ml hypoxanthine and 10% O + human serum. In order to ensure a microaerophilic environment, the parasites were maintained in a candle jar at 37 °C. Use of human erythrocytes to support the growth of P. falciparum was approved by the Internal Review Board of the Johns Hopkins University Bloomberg School of Public Health.

**Fast Performance Liquid Chromatography.** FPLC was performed using an ÄKTA Explorer system. Reverse-phase FPLC was conducted on the n-butanol extract of C. sp. Panama (resuspended in start buffer) using a RESOURCE RPC 3 mL (GE Healthcare Life Sciences) column under gradient elution between 2% and 85%
methanol in water 0.1% TFA at a constant 2 mL/min flow rate. Fraction F was collected, dried under vacuum, and the resulting residue resuspended in 20% DMSO. Size exclusion FPLC of this sample was performed on a Superdex Peptide 10/300 GL (GE Healthcare Life Sciences) column under isocratic elution with 20% DMSO in water at a constant flow rate of 1 mL/min.

**Plasmodium asexual stage growth inhibition assay.** Antiplasmodial activity of Chromobacterium spp. bacterial culture extracts against asexual stages of *P. falciparum* was assessed using a SYBR green I-based fluorescence assay as described earlier. Different concentrations of filtered bacterial culture extracts, their fractions or pure compound in 20% DMSO were dispensed in triplicate wells of 96 well microplates, followed by addition of synchronous ring stage *P. falciparum* cultures at 1% hematocrit and 1% parasitemia; parasites were synchronized using 5% Sorbitol as described previously. Chloroquine (250 nM) was used as positive control and 1% DMSO (i.e. final DMSO concentration) was used as negative control. After 72 h of incubation in a candle jar at 37 °C, equal volume of SYBR green-I solution (Invitrogen) in lysis buffer [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; w/v) and Triton X-100 (0.08%; v/v)] was added to each well and mixed gently and incubated for 1–2 h in the dark at room temperature. Plates were read on a fluorescence plate reader (Synergy HT, BioTek Instruments) with excitation and emission wavelengths of 485 and 535 nm, respectively. Percent inhibition was calculated relative to growth in negative (0% inhibition) and positive controls (100% inhibition). Results are shown as mean of at least 3 replicates ± standard deviation. For each treatment, significance was determined using a one-tailed one sample t-test to determine if it significantly lowered parasite growth compared to control. IC$_{50}$ values were estimated from a dose-response curve fitted to the normalized data using the least squares method with variable slope.

**Mass spectrometry.** Active fractions from FPLC purification were further resolved using ultra-performance liquid chromatography (UPLC) and analyzed by high-resolution electrospray mass spectrometry on a Waters Acquity Xeno-G2 (UPLC-ESI-MS). Samples were dissolved in 20% DMSO and separated using a BEH C18 column (Waters, 130 Å, 1.7 μm, 2.1 × 50 mm) at 0.3 mL/min with a gradient elution of 20% to 80% aqueous acetonitrile + 0.1% formic acid. MS and MS/MS spectra were collected in positive ion mode.

**Genome curation and comparisons.** Genomes were curated, and mining for secondary metabolite gene clusters was performed as described by Adamek, et al., with modifications. Genome sequences for nine different *Chromobacterium* species/strains were obtained from the sources listed in Table 4. Those at contig assembly level were run through MeDuSa against the sequence deposited for *C. violaceum* ATCC 12472 (NCBI RefSeq NC_005085.1), which was used as reference for further assembly. Pairwise genome average nucleotide identity (gANI) was calculated using the OrthoANIu algorithm, and the resulting similarity matrix was used to generate an UPGMA tree using DendroUPGMA.

The genomic sequence obtained for *C. sp. Panama* was then uploaded to antiSMASH v. 4. (bacterial) and the algorithm was run together with ClusterFinder for probabilistic detection of biosynthetic gene clusters. The uncurated clusters obtained were run through megaBLAST against each of the other eight *Chromobacterium* genomes and a similarity index was compounded for each query by multiplying query cover with % identity. A similarity index of 90% or above indicates that said *Chromobacterium* species/strains was present in the genome of that particular species; one of 30% or below indicates with confidence that the cluster is not replicated in the other genome.

**Romidepsin biosynthetic gene cluster analysis.** The romidepsin biosynthetic gene cluster in *C. sp. 968* (GenBank: EF210776.1) was used as reference for nucleotide sequence alignment in Geneious v5.4 (global alignment with free end gaps; cost matrix: 65% similarity) against the megalAST hit in each of the other *Chromobacterium* spp. genomes. Prodigal was run in each of these sequences to determine the boundaries of coding DNA sequences, and megalAST was used to assign their identity based on the original annotation in the 968 strain. Average pairwise sequence identity for coding sequences between the reference and other species was determined by averaging the pairwise identity values as returned by Geneious v5.4 for each of the genes in the cluster.

**Plasmodium gametocyte inhibition assay.** Gametocyte inhibition was measured as previously described. *P. falciparum* gametocyte cultures were initiated at 0.5% mixed stage parasitemia from low passage stock and cultures were maintained up to day 15 with daily media changes. At day 15 a blood smear was prepared and parasitemia was counted microscopically to calculate % mature stage V gametocytes. Gametocytes were then treated with increasing concentrations of romidepsin, 1 μM pyrvinium pamoate or 0.5% DMSO (vehicle control) for 48 hours. Gametocytes were then washed once in drug-free serum and infectious blood meals were prepared at 0.02% gametocytemia and fed to *A. gambiae* females as described below. Data from two biological replicates (each with three technical replicates) was analyzed by the Kruskal-Wallis test by comparing number of oocysts per midgut in treatment groups to those of the control and correcting for multiple comparison by the Dunn’s test.

**Anopheles gambiae rearing and Plasmodium infection assays.** *An. gambiae* Keele strain mosquitoes were obtained from the five year old rearing and infection at the John Hopkins University Malaria Institute. Plasmodium falciparum NF54 by allowing them to feed on stage V gametocyte cultures (0.02% gametocytemia; provided by the Johns Hopkins Malaria Institute Parasitology Core Facility) through artificial membrane feeders at 37 °C. Adult mosquitoes were starved for at least 4 h prior to feeding to guarantee robust feeding rates, and unfed
mosquitoes were removed from the cohort after feeding. Mosquitoes were then incubated for a further 7–8 days at 27°C and, to determine oocyst counts, midguts were dissected out in PBS, stained with 0.2% mercuricchrome and examined using a light–contrast microscope.

To study the influence of Chromobacterium spp. or romidepsin on P. falciparum infection of An. gambiae, female mosquitoes were provided with roughly 10^7 CFU/mL bacterial suspensions in 3% sucrose or romidepsin (AOBIOS, AOB1853) in 0.5% DMSO 3% sucrose at different concentrations, respectively. Oocyst numbers were determined as described above and compared to cohorts fed on vehicle alone; median values of a combination of at least 3 replicates are shown. Significance was determined using the Kruskal–Wallis test by comparing treatment groups to the control and correcting for multiple comparison by the Dunn's test.

**Chromobacterium spp. phylogenetic analysis.** 16S rRNA genomic sequences from the different chromobacteria (Table 4) in addition to C. sp. 968 (GenBank: EF210776.1) and other related bacterial species (Neisseria gonorrhoeae NCTC13800; Neisseria meningitidis LNP21362; Pseudomonas aeruginosa DSM 50071) were aligned in MEGA64 using ClustalW62. Evolutionary relationships between the sequences were inferred by the UPGMA method63; evolutionary distances were computed using the Maximum Composite Likelihood method64 and are shown in the units of the number of base substitutions per site.

**P. falciparum nuclear extracts and HDAC inhibition measurements.** Nuclear protein extracts of P. falciparum NF54 were obtained as described before65 following the modifications by Anne Hempel from the Manuel Linas group66. Briefly, P. falciparum NF54 cultures were pelleted (800 g, 5 min, low brake) and red blood cell lysis was promoted in 0.1% saponin solution; parasites were then pelleted (800 g, 10 min, low brake) and lysed in 20 mM HEPES, pH 7.8, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1% Triton X-100. Nuclei were harvested by centrifugation at 2,500 g, 5 min and nuclear proteins were extracted for 30 min using 20 mM HEPES, pH 7.8, 800 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 × protease inhibitor cocktail (Sigma, P8180). Debris was removed by centrifugation (13,000 g, 30 min) and nuclear protein extract was kept at −80°C in 15% glycerol until further use. HDAC inhibition by serial dilutions of romidepsin (AOBIOS, AOB1853) or n-butanol bacterial culture supernatants in 20% DMSO was assessed using the colorimetric EpiQuik HDAC Activity/Inhibition Assay Kit (EpiGenTek, P-4002) according to the manufacturer’s protocol and using 10 μg of these P. falciparum nuclear protein extracts as source of Plasmodium HDAC enzymes.

**Data availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

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Author Contributions

R.G.S., C.R.H.R., A.T., C.A.T., G.D. conceived experiments. R.G.S., C.R.H.R., A.T., Y.Q.C., J.B. performed experiments. R.G.S., C.R.H.R., C.A.T., G.D. analyzed data obtained. R.G.S., C.R.H.R., C.A.T., G.D. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

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