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Effect of clinically approved HDAC inhibitors on *Plasmodium*, *Leishmania* and *Schistosoma* parasite growth

Ming Jang Chua, Megan S.J. Arnold, Weijun Xu, Julien Lancelot, Suzanne Lamotte, Gerald F. Spath, Eric Prina, Raymond J. Pierce, David P. Fairlie, Tina S. Skinner-Adams, Katherine T. Andrews, 1, 1

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ABSTRACT

Malaria, schistosomiasis and leishmaniasis are among the most prevalent tropical parasitic diseases and each requires new innovative treatments. Targeting essential parasite pathways, such as those that regulate gene expression and cell cycle progression, is a key strategy for discovering new drug leads. In this study, four clinically approved anti-cancer drugs (Vorinostat, Belinostat, Panobinostat and Romidepsin) that target histone/lysine deacetylase enzymes were examined for in vitro activity against *Plasmodium knowlesi*, *Schistosoma mansoni*, *Leishmania amazonensis* and *L. donovani* parasites and two for in vivo activity in a mouse malaria model. All four compounds were potent inhibitors of *P. knowlesi* malaria parasites (IC₅₀ 9–370 nM), with belinostat, panobinostat and vorinostat having 8–45 fold selectivity for the parasite over human neonatal foreskin fibroblast (NFF) or human embryonic kidney (HEK 293) cells, while romidepsin was not selective. Each of the HDAC inhibitor drugs caused hyperacetylation of *P. knowlesi* histone H4. None of the drugs was active against *Leishmania* amastigote or promastigote parasites (IC₅₀ > 20 μM) or *S. mansoni* schistosomula (IC₅₀ > 10 μM), however romidepsin inhibited *S. mansoni* adult worm parings and egg production (IC₅₀ ~ 10 μM). Modest in vivo activity was observed in *P. berghei* infected mice dosed orally with vorinostat or panobinostat (25 mg/kg twice daily for four days), with a significant reduction in parasitemia observed on days 4–7 and 4–10 after infection (P < 0.05), respectively.

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1. Introduction

Tropical parasitic diseases cause significant morbidity and mortality, infecting hundreds of millions of people globally, particularly in developing countries (Lozano et al., 2012; Murray et al., 2012). In 2015 alone there were ~214 million clinical cases of malaria and 438,000 deaths associated with this disease (World Health Organization). On an annual basis 1–2 million and ~250 million people are reported to be infected with *Leishmania* (World Health Organization, 2008; Pigott et al., 2014) and *Schistosoma* parasites, respectively (Colley et al., 2014). Although drugs for each of these parasitic infections are available, prevention and treatment is often difficult due to side-effects (Sundar and Chakravarty, 2015) or ineffective due to drug-resistant parasites (Croft et al., 2006; Dondorp et al., 2009; Dondorp and Ringwald, 2013; Berg et al., 2015; Takala-Harrison et al., 2015). There is no vaccine that is clinically available or widely effective for any of the human parasitic diseases. Thus, the discovery of novel drug targets, and new chemotherapies with novel mechanisms of action, are high priorities. Small molecules that act on epigenetic regulatory proteins, such as those responsible for post-translational modifications of histones, are of increasing interest as chemical tools for dissecting fundamental mechanisms of parasite growth and as possible new drug leads (Andrews et al., 2012b; Ay et al., 2015; Cheeseman and Weitzman, 2015). Clinically approved drugs are also attracting
interest for repurposing for new uses since this can shorten time to market and reduce costs compared to de novo drug discovery for malaria or Neglected Tropical Diseases (NTDs). Alternatively, they may be used as new starting points for the rational development of parasite targeting compounds (Andrews et al., 2014).

Histone deacetylases (HDACs) are now known to target both histone proteins and many non-histone proteins and thus are sometimes described as lysine deacetylases (KDACs). Histone/lysine deacetylases and acetyltransferases, respectively, remove and add acetyl groups from histones and other proteins, just as corresponding demethylases and methyltransferases remove and add methyl groups to lysine sidechains of proteins (Arrowsmith et al., 2012). These posttranslational modifications contribute to the regulation of numerous essential biological processes in eukaryotes including transcriptional regulation (Heintzman et al., 2009), cell cycle progression (Montenegro et al., 2015) and apoptosis (Bose et al., 2014; Zhang and Zhong, 2014). Aberrant expression of these proteins is a feature of some human diseases, such as cancers, making these epigenetic regulatory enzymes “drugable” targets (Arrowsmith et al., 2012; Falkenberg and Johnstone, 2014; Brien et al., 2016). Likewise, some epigenetic regulatory proteins have been shown to play essential roles in proliferation and life cycle stage progression of parasitic pathogens (Azizi et al., 2009; Coleman et al., 2014), with the proteins having low homology to human proteins (Andrews et al., 2012b, 2012c) or significant differences in important catalytic domains (Marek et al., 2013; Melesina et al., 2015) that make them attractive anti-parasitic drug targets. HDAC homologues have been identified in all major human parasitic pathogens and different classes of HDAC inhibitors have also been shown to have activity against some of these parasites, including Plasmodium species that cause malaria and the causative agents of selected NTDs including Leishmania and Schistosoma parasites (reviewed in (Andrews et al., 2012b, 2012c; Marek et al., 2015)).

Several HDAC inhibitors have been clinically approved for human use for different cancers and these drugs are potential leads for drug targets. HDAC homologues have been identified in all major human parasitic pathogens and different classes of HDAC inhibitors have also been shown to have activity against some of these parasites, including Plasmodium species that cause malaria and the causative agents of selected NTDs including Leishmania and Schistosoma parasites (reviewed in (Andrews et al., 2012b, 2012c; Marek et al., 2015)).

2. Materials and methods

2.1. Compounds

Vorinostat (SAHA; Sigma Aldrich, USA), Romidepsin (FK228; Istodax; Selleck Chemicals, USA), and Belinostat (PXD101; Beleodaq; Spectrum Pharmaceuticals, Inc., USA) are approved for cutaneous or peripheral T-cell lymphoma (Grant et al., 2007; Prince and Dickinson, 2012; Thompson, 2014), while Panobinostat (LBH-589; Selleck Chemicals, USA) is approved for combination therapy of multiple myeloma (Garnock-Jones, 2013). Two days later, resazurin was added (10 μl per well) to stain macrophage nuclei (Hoechst 33342) and parasites growing in cytoplasm were imaged with a fluorescent mCherry reporter using pLEXSY-cherry-sat2 vector (Jena Bioscience) and an inverted microscope. Images were acquired at 5× magnification (excitation 558 ± 10 nm). Following background subtraction (complete parasite culture medium with toxic cycloheximide (50 μg/mL)) and Hoechst 33342 (5 μg/mL) fluorescence intensity was measured 24 h after resazurin addition using a Tecan Sa 2 reader (excitation 558 ± 4.5 nm; emission 585 ± 10 nm). Following background subtraction (complete parasite culture medium with resazurin without parasites), data were expressed as percentage growth of DMSO-treated controls. For HCA, mouse bone marrow-derived macrophages were infected with lesion-derived L. amazonensis amastigotes using a High-Content phenotypic Assay (HCA) (Aulner et al., 2015). For the dye reduction assay, compounds were tested in quadruplicate at 20, 4 and 0.8 μM at 26 °C and 37 °C for promastigotes and amastigotes, respectively. Briefly, parasites growing in logarithmic phase (5 × 10⁶/well) were seeded in 384-well plates containing compound dilutions and controls including DMSO vehicle and amphotericin B (0.5 μM). Two days later, resazurin was added (10 μL per well at 25 μg/mL) and fluorescence intensity was measured 24 h after resazurin addition using a Tecan Safire 2 reader (excitation 558 ± 4.5 nm; emission 585 ± 10 nm). Following background subtraction (complete parasite culture medium with resazurin without parasites), data were expressed as percentage growth of DMSO-treated controls. For HCA, mouse bone marrow-derived macrophages were infected with lesion-derived L. amazonensis amastigotes. These parasites were genetically modified by chromosomal integration of the fluorescent mCherry molecule using pLEXSY-cherry-sat2 vector (Jena Bioscience) and propagated in Swiss nu/nu mice to keep virulence feature. One day after macrophage infection, compounds were added in quadruplicates at 10 or 1 μM final concentration for 3 days. Controls included DMSO vehicle, anti-leishmanial amphotericin B (1 μM) and cytoxic cycloheximide (50 μg/mL). Fluorescent reporters were added for 1 h to stain macrophage nuclei (Hoechst 33342) and parasite vacuoles (LysoTracker Green DND26) and images of living macrophage cultures were acquired using the automatic Opera QHS confocal reader (Perkin Elmer Technology). Images

Porinostat (SAHA and chloroquine dihydrophosphate salt were purchased from Sigma-Aldrich (USA). Romidepsin (FK228), Belinostat (PXD101), and Panobinostat (LBH589) were purchased from Selleck Chemicals. All HDAC inhibitors were prepared as 10–20 mM stock solutions in 100% DMSO. Chloroquine was prepared as a 10 mM stock in phosphate buffered saline (PBS).

2.2. Plasmodium in vitro growth inhibition assays

P. knowlesi A1H1 (Moon et al., 2013) and P. falciparum 3D7 parasites were cultured in O positive human erythrocytes in RPMI 1640 media (Gibco, USA) supplemented with 10% heat-inactivated pooled human sera (AB for P. knowlesi) and 5 μg/mL gentamicin, as previously described (Trager and Jensen, 1976; Moon et al., 2013). P. knowlesi culture media also included 50 μg/mL hypoxanthine and 5 μL/Albamax II. In vitro activity of drugs was determined using previously described ³H-hypoxanthine incorporation assays for P. knowlesi A1H1 (Arnold et al., 2016) and P. falciparum (Skinner-Adams et al., 2007). Briefly asynchronous Plasmodium infected erythrocytes (0.25% parasitemia and 2% haematocrit for P. knowlesi; 1% parasitemia and 1% haematocrit for P. falciparum) were seeded into 96-well tissue culture plates, with test compounds or controls, in hypoxanthine-free culture media. Chloroquine was used as a positive control in all assays. For P. knowlesi, after incubating for 24 h, 0.5 μCi ³H-hypoxanthine (PerkinElmer, USA) was added to each well and cells were cultured for a further 24 h and then harvested onto 1450 MicroBeta filter mats (Wallac, USA). For P. falciparum, 0.5 μCi ²H-hypoxanthine was added at the start of the assay and after 48 h incubation cells were harvested as above. In each case ³H-hypoxanthine incorporation was determined using a 1450 MicroBeta liquid scintillation counter (PerkinElmer, USA) and percentage inhibition of growth determined compared to matched 0.5% DMSO vehicle controls included in each assay plate. Each independent experiment was carried out in triplicate and performed at least three times. 50% inhibitory concentrations IC⁵₀(s) were determined via log linear interpolation (Huber and Koella, 1993).

2.3. Leishmania growth inhibition assays

L. donovani parasites (MHOM/SD/62/1S-CL2D) were cultured in modified M199 media as previously described (Pescher et al., 2011). Lesion-derived amastigotes of L. amazonensis (MPRO/BR/1972/ M1841) were used for macrophage infection or differentiated into promastigotes in L. donovani promastigote medium (Pescher et al., 2011). Cell-cycling promastigotes of both Leishmania species were taken from the logarithmic growth phase for viability assays. Anti-leishmanial activity of compounds was evaluated against host cell-free parasites using a resazurin reduction assay (adapted from (Durieu et al., 2016)) and on intramacrophagic L. amazonensis amastigotes using a High-Content phenotypic Assay (HCA) (Aulner et al., 2013). For the dye reduction assay, compounds were tested in quadruplicate at 20, 4 and 0.8 μM at 26 °C and 37 °C for promastigotes and amastigotes, respectively. Briefly, parasites growing in logarithmic phase (5 × 10⁶/well) were seeded in 384-well plates containing compound dilutions and controls including DMSO vehicle and amphotericin B (0.5 μM). Two days later, resazurin was added (10 μL per well at 25 μg/mL) and fluorescence intensity was measured 24 h after resazurin addition using a Tecan Safire 2 reader (excitation 558 ± 4.5 nm; emission 585 ± 10 nm). Following background subtraction (complete parasite culture medium with resazurin without parasites), data were expressed as percentage growth of DMSO-treated controls. For HCA, mouse bone marrow-derived macrophages were infected with lesion-derived L. amazonensis amastigotes. These parasites were genetically modified by chromosomal integration of the fluorescent mCherry molecule using pLEXSY-cherry-sat2 vector (Jena Bioscience) and propagated in Swiss nu/nu mice to keep virulence feature. One day after macrophage infection, compounds were added in quadruplicates at 10 or 1 μM final concentration for 3 days. Controls included DMSO vehicle, anti-leishmanial amphotericin B (1 μM) and cytoxic cycloheximide (50 μg/mL). Fluorescent reporters were added for 1 h to stain macrophage nuclei (Hoechst 33342) and parasite vacuoles (LysoTracker Green DND26) and images of living macrophage cultures were acquired using the automatic Opera QHS confocal reader (Perkin Elmer Technology). Images
were analysed using Acapella™ and both anti-leishmanial activity and toxicity to host cells were determined for each compound.

2.4. *S. mansoni* viability assays

A resazurin-based fluorescence assay was used to determine the inhibitory activity of compounds against Newly-Transformed *S. mansoni* Schistosomula (NTS) (Marxer et al., 2012), as previously described (Heimburg et al., 2016). Negative and positive controls, including untreated schistosomula, killed larvae (70% ethanol) and schistosomula exposed to praziquantel (PZQ) were included in each assay. The stability of adult worm pairs and egg laying in culture were measured as previously described (Vanderstraete et al., 2013). All assays were performed in triplicate on two separate occasions.

2.5. Protein hyperacetylation assays

*P. knowlesi* protein hyperacetylation assays were carried out as previously described for *P. falciparum* (Sumanadasa et al., 2012; Trenholme et al., 2014). Briefly, trophozoite stage infected-erythrocytes were incubated with test compounds (1× or 5× IC50), chloroquine negative control, or vehicle control (0.2% DMSO) for 3 h under standard culture conditions. Cells were then pelleted, lysed with 0.15% saponin and the resulting parasite pellets washed with PBS before resuspension in 1× SDS-PAGE loading dye. Following heat denaturation (94 °C, 3 min) protein was analysed by SDS-PAGE and Western blot using anti-(tетra) acetyl histone H4 antibody (1:2000 dilution; Millipore; 06–866) and goat anti-rabbit—594 dye secondary antibody (Alexa Fluor®). Anti-(tетra) acetyl histone H4 antibody is reported by the manufacturer to recognize acetylated forms of histone H4 and to cross-react with acetylated histone H2B and possibly other acetylated histones. Anti-(tетra) acetyl histone H4 antibody has previously been validated for *P. falciparum* (e.g (Sumanadasa et al., 2012; Engel et al., 2015)) (PlasmoDB gene ID PF3D7_1105000 (Aurrecoechea et al., 2009)) and the *P. knowlesi* H4 amino acid sequence (PlasmoDB gene ID PKNH_0902600 (Aurrecoechea et al., 2009)) is identical. Membranes were imaged using an FLA-5000 imaging system (FUJIFILM, USA). As an appropriate antibody loading control was not available at the time this work was carried out (those normally used in this laboratory for *P. falciparum* did not cross-react with this species; data not shown), protein loading was assessed by Coomassie blue-staining of samples separated by SDS-PAGE.

2.6. Homology modelling and docking

As there is no reported crystal structure of HDAC(s) from any *Plasmodium* species, a homology model of the PkHDAC1 was built using the homology modelling suite in Maestro (Schrödinger Release, 2016–1). The BLAST search engine within the suite was used for template searching before model construction. Human HDAC2 (pdb code: 3MAX) was chosen as the most suitable template due to its overall sequence identity (62%) and similarity (81%) with the PkHDAC1 protein, and its high resolution (2.1 Å) crystal structure in complex with a small molecule inhibitor. The energy-based approach was used to build the final model. The homology model created from Maestro was checked for model quality and stereochemistry using SwissModel (Kiefel et al., 2009). For ligand docking experiments, 2D-structures of vorinostat, panobinostat and belinostat were drawn in ChemBioDraw H4.0 and saved as sdf files. The 3D coordinates of the ligands were prepared in Maestro using the Ligand Preparation suite at physiological pH using the OPLS (2005) force field. GOLD (version 5.2.2) was used for ligand docking and Chemscore for scoring the relative affinities of ligand poses. Ligands were docked in the active site defined by a 15 Å radius around the OD2 atom of Asp 262. Each ligand was docked 20 times using scaffold constraint settings to ensure that each hydroxamate mimicked the native binding mode of vorinostat bound in the crystal structure human HDAC2 (pdb code: 4LXZ) during ligand docking simulations.

2.7. In vivo anti-*Plasmodium* efficacy studies

In vivo anti-*Plasmodium* activity was examined using groups of six female BALB/c mice (6–8 weeks old; Animal Resources Centre, Perth, Australia) infected via intra-peritoneal (i.p.) injection with 105 *P. berghei* QIMR (Saul et al., 1997) infected erythrocytes taken from an infected passage mouse. Mice were treated by oral gavage with 100 µl test compounds (25 mg/kg) or vehicle control (50% DMSO in PBS) twice daily for four days, beginning 2 h post infection (p.i.) and with a 4 h interval between doses (as published for the hydroxamate HDAC inhibitor SB939 (Sumanadasa et al., 2012)). Chloroquine (10 mg/kg in PBS) was a positive control administered via oral gavage twice daily for three days beginning 2 h p.i. Peripheral parasitemia was monitored daily from day 4 p.i. by microscopic examination of Giemsa-stained thin blood smears prepared from tail snip bleeds. Mice were euthanized according to an approved scorecard of criteria and all animal work was conducted using protocols approved by National Health and Medical Research Council (NHMRC) of Australia Animal Code of Practice, as approved by the Griffith University Animal Ethics committee. Data were analysed using two-tailed Student’s t-Test using Graph Pad Prism (version 5).

3. Results and discussion

Targeting epigenetic regulatory enzymes to combat parasitic diseases is of growing interest (Andrews et al., 2012b, 2012c; Marek et al., 2015). However, progress on HDAC inhibitors for use in the parasite field lags behind cancer research where HDAC(s) have been targets for clinically approved drugs since 2007 (Grant et al., 2007). New HDAC inhibitors that have been developed for cancer therapy are therefore of potential interest in the anti-parasitic drug discovery arena, either from a potential repurposing approach or as starting points for discovery of parasite-selective inhibitors. Here the comparative anti-parasitic profiles of four HDAC inhibitors (Vorinostat, Romidepsin, Belinostat and Panobinostat) were assessed in *in vitro* against the zoonotic *P. knowlesi* malaria parasite species, leishmania parasites (*L. amazonensis* and *L. donovani*) and schistosomal parasites (*S. mansoni*). Two of the compounds were also examined *in vivo* in a murine model of malaria.

Until recently, in the malaria field the ability to easily and rapidly perform *in vitro* drug testing on *Plasmodium* species was limited to *P. falciparum* (Trager and Jensen, 1976). However, the adaptation of the zoonotic *P. knowlesi* species to continuous *in vitro* culture in human erythrocytes (Lim et al., 2013; Moon et al., 2013; Gruning et al., 2014) and modification of the standard 3H-hypoxanthine-uptake assay method for *P. knowlesi* (Arnold et al., 2016) has changed this situation, allowing the four clinically used HDAC inhibitors to be tested against *P. knowlesi* for the first time. All four compounds are sub-micromolar inhibitors of *P. knowlesi* growth (IC50 9–370 nM), and as for *P. falciparum* (included as a control; Table 1) the most potent compound against *P. knowlesi* was Panobinostat (Table 1; IC50 9 (±1) nM). These data extend the human-malaria inhibitors to *Plasmodium* species targeted by HDAC inhibitors *in vitro* or *ex vivo* to include *P. falciparum* (Engel et al., 2015), *P. vivax* (Marfurt et al., 2011) and now *P. knowlesi*. A comparison of the *P. knowlesi* and *P. falciparum* IC50 values with cytotoxicity against human Neutotal Foreskin Fibroblast (NFF) and Human Embryonic Kidney
(HEK 293) cells (Engel et al., 2015) demonstrated that the three hydroxamate-based HDAC inhibitors Vorinostat, Panobinostat and Belinostat are modestly selective for Plasmodium parasites (Table 1; $SI < 1$). In contrast to the activity observed against malaria parasites, no activity was observed for any compound against L. amazonensis promastigotes (Fig. 1A), and when assessed at 1 mM or 10 mM against intramacrophagic amastigotes all compounds were either toxic to macrophages or had no anti-leishmanial activity (data not shown). Only weak activity was observed for belinostat, panobinostat and vorinostat against free L. donovani promastigotes (20–35% inhibition at 20 mM; Fig. 1A), with no growth inhibition observed for romidepsin (Fig. 1A). Likewise, poor activity was observed for all compounds against L. donovani axenic amastigotes (Fig. 1A). No anti-leishmanial activity was seen at concentrations below 20 mM for either Leishmania species at all developmental stages examined (data not shown). The activity of these drugs against S. mansoni was more variable (Fig. 1B). While the hydroxamic acid HDAC inhibitors were generally poor inhibitors of S. mansoni, panobinostat demonstrated modest inhibitory activity against adult worm pairing (30–40% at 10 mM; $P = 0.001$) and egg production (<50% at 10 mM; $P = 0.008$; Fig. 1B). In the same assay conditions PZQ (10 mM) induced the death of adult worms (and in consequence abolished pairing and egg-laying) but only very weakly affected the viability of schistosomula (8% viability observed).
reduction; P > 0.05; data not shown), consistent with previous observations (Panic et al., 2015). In addition, at 10 μM the cyclic tetrapeptide Romidepsin completely inhibited adult worm pairing and egg production (Fig. 1B), an inhibitory activity that was also seen at 1 μM and accompanied by tegumental damage (data not shown). The greater effect of romidepsin on adult worm pairing and egg production as compared to the other inhibitors may be linked to the fact that romidepsin is a prodrug, requiring intracellular reduction to generate a reactive sulfhydryl group that interacts with the zinc ion in the HDAC catalytic pocket (Furumai et al., 2002). Previous studies have reported poor activity for vorinostat (used as a control in this work) against Leishmania and Schistosoma (Dubois et al., 2009; Patil et al., 2010) and this was hypothesized to be due to protection mechanisms such as higher efflux or lower influx of compounds, poor cell permeability or low levels of target proteins (Melesina et al., 2015), although none of these mechanisms have been confirmed.

The effects of the clinical HDAC inhibitors on P. knowlesi was further investigated using Western blot, with all drugs causing hyperacetylation of P. knowlesi histone H4 (Fig. 2), indicating inhibition of PkHDAC activity, either directly or indirectly. Hyperacetylation assays showed four distinct bands ranging from ~11 kDa (the expected size of H4) to ~16 kDa and relative density analysis showed a hyperacetylation effect using only the ~11 kDa band or all bands combined (Fig. 2B). The higher molecular weight bands likely correspond to hyperacetylated forms of H2B/H2Bv (~13–14 kDa) and H2AZ (~16 kDa) (Miao et al., 2006) as this antibody is reported to cross-react with acetylated forms of histones other than H4 (see Section 2.5). The hyperacetylation effect observed here for the first time in P. knowlesi is consistent with that previously reported for these and other HDAC inhibitors (Miao et al., 2006; Dow et al., 2008; Trenholme et al., 2014; Engel et al., 2015) against P. falciparum and is considered a marker of HDAC inhibition in the parasite (e.g. Darkin-Rattray et al., 1996; Andrews et al., 2008, 2012a; Chaal et al., 2010). In P. falciparum, three class I/II HDAC homologues (encoded by PfHdac1, PfHdac2 and PfHdac3 genes) have been annotated in the PlasmoDB (Aurrecoechea et al., 2009) genome database. Two additional class III homologues are also present (Aurrecoechea et al., 2009), but are not essential in asexual intraerythrocytic stage parasites (Freitas-Junior et al., 2005; Tonkin et al., 2009). While recombinant forms of PfHdac2 and PfHdac3 are not currently available, the activity of PfHdac1 can be inhibited by anti-Plasmodial HDAC inhibitors (Patel et al., 2009). Although no recombinant PfHdac3 are available to assess direct enzyme inhibition, it is likely that a PfHdac is a target of these anti-cancer HDAC inhibitor compounds in P. knowlesi. Homologues of each of the P. falciparum class I/II HDACs, (amino acid sequence identities ranging from 43% to 95%; Supplemental Table S1) are annotated in the P. knowlesi genome (Aurrecoechea et al., 2009). The highest homology is between PfHdac1 and its homologue PkHdac1 (95%), a finding which fits well with our Western blot data demonstrating that PfHdac1 polyclonal antibody (Trenholme et al., 2014; Engel et al., 2015) cross-reacts with P. knowlesi protein lysates (Supplemental Figure S1).

As PfHdac1 (PlasmoDB gene ID PF3D7_0925700) is inhibited by these clinically approved HDAC inhibitors (Engel et al., 2015), a three dimensional homology structural model of PkHdac1 was generated (Fig. 3A) to examine the predicted binding mode of these ligands to the P. knowlesi homologue (PkHdac1; PlasmoDB (Aurrecoechea et al., 2009) gene ID Pkh_072280). The model of PkHdac1, which was almost identical to that for PfHdac1 (Wheatley et al., 2010; Sumanadasa et al., 2012), adopted a canonical HDAC fold. A Ramachandran plot of the homology model (Supplemental Figure S2) showed 91.2% and 8.2% of residues from the model located in most favoured or allowed regions, respectively. The QMEANscore6 and dfire_energy of the model was 0.75 and –599.2, indicating that the homology model was stereochemically favourable and energetically similar to native protein structures. For comparison, amino acids that differ between P. falciparum and P. knowlesi (corresponding to sequence comparison in Supplemental Figure S1) are coloured in red. The three HDAC inhibitors that showed some selectivity for P. knowlesi versus mammalian cells (vorinostat, panobinostat, belinostat) were docked into the PkHdac1 model to investigate possible binding modes within the enzyme active site (Fig. 3B). All ligands showed their hydroxamate bound to zinc in the active site, with the linker occupying the active site tunnel of the enzyme and making hydrophobic and van der Waals interactions with residues Tyr301, Phe148, Phe203. The terminal group at the non-hydroxamate end of each inhibitor varied in the orientation towards the loop residues
oral gavage twice daily with 25 mg/kg panobinostat (pirtifluzal) and peripheral blood parasitemia was also observed for mice treated by only control mice (Fig. 4B). The survival (based on euthanizing in mean peripheral parasitemia on these days compared to vehicle-4) and p reduction in peripheral blood parasitemia was observed for mice treated according to ethics approved scorecard criteria) of mice treated with panobinostat was also significantly improved compared to the vehicle control group (p = 0.0013; Fig. 4D). Mice treated orally with the antimalarial drug chloroquine (10 mg/kg; twice daily for four days beginning 2 h p.i.) did not develop detectable peripheral blood parasitemia for the duration of the experiment (data not shown). While additional studies would be required to investigate why the in vivo activity of panobinostat is greater than for vorinostat in the mouse malaria model, panobinostat does have a 40–60 fold greater inhibitory potency against Plasmodium parasites in vitro (Table 1). However the direct in vivo activity of these drugs against P. berghei parasites is not known and pharmacokinetic properties are also likely to play a role. For example, preclinical studies in mice dosed orally with 50 mg/kg vorinostat or panobinostat have shown that these drugs have substantially different pharmacokinetic profiles, with panobinostat having a >2-fold longer half-life (Table 2) (Yeo et al., 2007). While these in vivo data suggest that vorinostat has limited anti-Plasmodium activity, the data obtained for panobinostat may be of interest given the reported improvement in pharmacokinetics for this drug in humans versus mice. Panobinostat has an approximately five-fold greater oral bioavailability (F%) in humans compared to mice with a 6–10 fold greater half-life (t1/2: 2.9 h and 16–30 h; Table 2) (Yeo et al., 2007). Humans receiving a single oral dose of panobinostat (at the recommended daily dose for cancer patients of 20 mg) have a reported mean Cmax of 24.3 (±12) ng/mL (~70 nM) [European Medicines Agency, 2015], >7-fold higher than the in vitro IC50 of this compound against Plasmodium parasites. However, as adverse effects are commonly reported at this dose (FARYDAK® product sheet (Novartis)) repurposing panobinostat for malaria would be difficult to justify. Nevertheless, these data raise the possibility of developing panobinostat analogues with similar pharmacokinetic profiles but improved Plasmodium-specific potency and selectivity.

of the protein. The most potent inhibitor (panobinostat) of P. knowlesi made π-π interactions between its terminal indole ring and Phe203. Interestingly, the loop spanning residues 91–99 in both PkHDAC1 and PfHDAC1 have two extra residues (Ala95, Thr96) compared to human HDAC2. This repositions the sidechain of conserved loop residue Asp97, pushing it further into solvent and the backbone carbonyl oxygen of Thr96 occupies the location of the Asp97 sidechain of human HDAC2. As a result, the free amine group of panobinostat makes a hydrogen bond with the backbone carbonyl oxygen atom of Thr96. Previous modelling of PfHDAC1 suggested that Asp97 is in a position similar to hHDAC8 (Wheatley et al., 2010). More interestingly, panobinostat docked in the current model adopted a G-shaped binding mode (Fig. 3B), which is similar to the findings from Melesina et al. (2015) reporting that G-shaped compounds are more favourable for selective inhibitors of parasitic HDACs. These modelling results provide support for binding to, and inhibition of, both PkHDAC1 and PfHDAC1 by these drugs and also rationalizes the stronger inhibition observed for panobinostat.

Given the in vitro activity of the anti-cancer HDAC inhibitors against Plasmodium, we extended our in vitro findings to investigate for the first time in vivo antimalarial efficacy of vorinostat and panobinostat using a mouse model of malaria. A significant reduction in peripheral blood parasitemia was observed for mice treated by oral gavage twice daily with 25 mg/kg vorinostat compared to vehicle-only control mice (p < 0.01 (day 4–6) and p < 0.05 (day 7)), however this only resulted in a one day delay in parasitemia progression (Fig. 4A). A significant reduction in peripheral blood parasitemia was also observed for mice treated by oral gavage twice daily with 25 mg/kg panobinostat (p < 0.05 (day 4) and p < 0.01 (day 5–10), corresponding to a 2–3 fold reduction in mean peripheral parasitemia on these days compared to vehicle-only control mice (Fig. 4B). The survival (based on euthanizing according to ethics approved scorecard criteria) of mice treated
received 100 thin blood smears. Panels A and C show mean number of parasites per 100 erythrocytes (mean % parasitemia; solid black line) or panobinostat (B and D; dashed line) versus control group (solid black line). Panels A and B show mean number of parasites per 100 erythrocytes (mean % parasitemia; >800 erythrocytes counted for each mouse) for 6 mice per treatment group (dashed lines) versus control group (solid black lines). Panels C and D show survival rates (mice were euthanized when parasitemia reached ~25%) for treated (dashed lines) versus DMSO vehicle-only control (solid black lines) mice.

**Fig. 4. In vivo activity of orally administered vorinostat and panobinostat in Plasmodium berghei infected BALB/c mice.** Female 6–8 week old BALB/c mice (n = 6) were injected i.p. with 10^6 P. berghei QMIR infected erythrocytes. Mice were treated by oral gavage with 25 mg/kg vorinostat (A and C; dashed line) or panobinostat (B and D; dashed line) twice daily for four days beginning 2 h post infection, with 4 h between first and second dose (100 μL/dose diluted freshly in 10% DMSO in PBS). Control mice (A-D; solid black line) received 100 μL vehicle only (10% DMSO in PBS) under the same dosing schedule. Parasitemia was monitored daily starting day 4 p.i. via microscopic examination of Giemsa-stained thin blood smears. Panels A and B show mean number of parasites per 100 erythrocytes (mean % parasitemia; >800 erythrocytes counted for each mouse) for 6 mice per treatment group (dashed lines) versus control group (solid black lines). Panels C and D show survival rates (mice were euthanized when parasitemia reached ~25%) for treated (dashed lines) versus DMSO vehicle-only control (solid black lines) mice.

### Table 2

Pharmacokinetic parameters of oral vorinostat and panobinostat in mice and humans.

| Parameter   | Mice  | Humans |
|-------------|-------|--------|
|             | vorinostat | panobinostat | vorinostat | panobinostat |
| AUC0→∞ (ng·h/mL) | 619 | 126 | 1698 | 183–373 |
| t1/2 (h)    | 0.75 | 2.90 | 1.5 | 16–30 |
| Cmax (ng/mL) | 501 | 116 | 658 | 23–71 |
| F (%)       | 8.33 | 4.62 | 43 | 21 |

* Female BALB/c nude mice (18–22 g; 8–10 weeks old) dosed orally with 50 mg/kg vorinostat or panobinostat (Yeo et al., 2007).

* Human subjects administered 200–600 mg oral vorinostat (Kavanaugh et al., 2010).

* Human subjects administered 15–80 mg oral panobinostat (Prince et al., 2009) and references therein).

* Human subjects administered 15–80 mg oral panobinostat (Prince et al., 2009) and references therein).

* Mean % Survival

* Mean % Parasitemia

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