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

Ming Jang Chua [a], Megan S.J. Arnold [a], Weijun Xu [b], Julien Lancelot [c], Suzanne Lamotte [d], Gerald F. Späth [d], Eric Prina [d], Raymond J. Pierce [c], David P. Fairlie [b, 1], Tina S. Skinner-Adams [a, 1], Katherine T. Andrews [a, *, 1]

[a] Griffith Institute for Drug Discovery, Griffith University, Queensland, Australia
[b] Institute for Molecular Bioscience, The University of Queensland, Brisbane, 4072, Australia
[c] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 8204- CIIL -Centre D'Infection et D'Immunité de Lille, F-59000 Lille, France
[d] Institut Pasteur and INSERM U1201, Unité de Parasitologie Moléculaire et Signalisation, Paris, France

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ABSTRACT

Malaria, schistosomiasis and leishmaniases 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 (IC_{50} 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 (IC_{50} > 20 μM) or *S. mansoni* schistosomula (IC_{50} > 10 μM), however romidepsin inhibited *S. mansoni* adult worm parings and egg production (IC_{50} ~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.

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

* Corresponding author. Griffith Institute for Drug Discovery, Don Young Road, Building N.75, Griffith University, Nathan, Queensland, Australia 4111.
E-mail address: k.andrews@griffith.edu.au (K.T. Andrews).
[1] These authors contributed equally.

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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 "druggable" 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 parasite pathogens (Azzi 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 application to parasitic diseases. 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, 2015). In this study, we assessed the capacity of all four drugs to inhibit the growth of parasites that cause malaria (*P. knowlesi*), leishmaniasis (*L. amazonensis* and *L. donovani*) and schistosomiasis (*S. mansoni*). We also investigated the *in vivo* antimalarial potential of orally administered vorinostat and panobinostat in a murine model of malaria.

2. Materials and methods

2.1. Compounds

Vorinostat (SAHA) and chloroquine diphosphate 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 10–20 mM stock in phosphate buffered saline (PBS).

2.2. Plasmodium in vitro growth inhibition assays

P. knowlesi A1H.1 (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 g/L Albumax II. *In vitro* activity of drugs was determined using previously described ^{3}H-hypoxanthine incorporation assays for *P. knowlesi* A1H.1 (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 ^{3}H-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 ^{3}H-hypoxanthine was added at the start of the assay and after 48 h incubation cells were harvested as above. In each case ^{3}H-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 IC_{50}(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 (5×10^{4}/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 HCAs, 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 cytotoxic cycloheximide (50 μg/mL). Fluorescent reporters were added for 1 h to stain macrophage nuclei (Hoechst 33342) and parasitophorous vacuoles (LysoTracker Green DND26) and images of living macrophage cultures were acquired using the automatic Opera QEHS confocal reader (Perkin Elmer Technology). Images