

## Discovery of a Selective Series of Inhibitors of *Plasmodium falciparum* HDACs

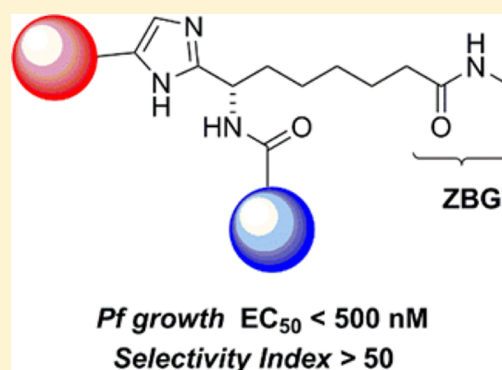
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### S Supporting Information

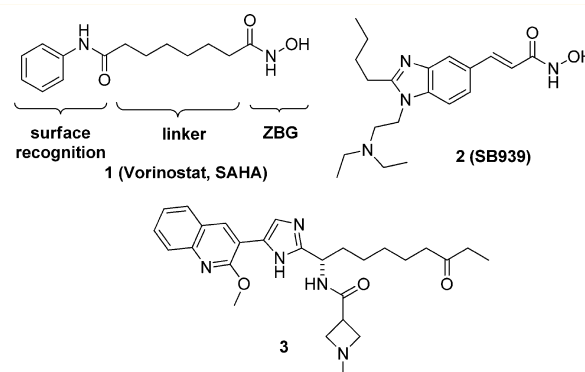
**ABSTRACT:** The identification of a new series of *P. falciparum* growth inhibitors is described. Starting from a series of known human class I HDAC inhibitors a SAR exploration based on growth inhibitory activity in parasite and human cells-based assays led to the identification of compounds with submicromolar inhibition of *P. falciparum* growth ( $EC_{50} < 500$  nM) and good selectivity over the activity of human HDAC in cells (up to >50-fold). Inhibition of parasital HDACs as the mechanism of action of this new class of selective growth inhibitors is supported by hyperacetylation studies.



**KEYWORDS:** Malaria, *Plasmodium falciparum*, PfHDAC1, 4-arylimidazoles

Infection with malaria parasites such as *Plasmodium falciparum* remains a devastating cause of death in tropical geographies with 40% of the world population at risk of acquiring the disease. There are approximately 200 million clinical cases of malaria every year leading to an estimated 600,000 deaths.<sup>1</sup> The requirement for improved therapies to treat and to cure malaria is an evident medical and humanitarian need that is exacerbated by an alarming rise in parasite resistance to the current standard of care.<sup>2,3</sup> Drugs that operate via novel mechanisms of action for which no innately resistant parasites are expected are therefore especially desirable.

DNA is tightly packed around histone proteins in the nucleus of eukaryotic cells with its transcription being regulated by chemical modifications to the nucleosomal histone proteins themselves. Histone deacetylases (HDACs) are zinc-dependent enzymes that play crucial roles in modulating mammalian cell chromatin structure, transcription, and gene expression.<sup>4–6</sup> HDACs have also been identified as important regulators of transcription in *P. falciparum*,<sup>7–10</sup> and inhibition of *P. falciparum* histone deacetylases (PfHDACs) has been reported to both effectively kill the parasites (Vorinostat, Figure 1)<sup>11–16</sup> and lead to efficacy in animal models of malaria (compound 2).<sup>17</sup> Such findings underscore the potential for PfHDAC inhibitors to be used for malaria therapy.<sup>18–20</sup>



**Figure 1.** Structures of known human HDAC inhibitors.

Of the five HDAC encoding genes known in *P. falciparum* one has homology to mammalian class I isoforms (PfHDAC1), two are similar class II (PfHDAC2 and 3) mammalian HDACs, while the remaining two are class III HDACs, or silent information regulator 2 (SIR2) proteins.<sup>19</sup> In light of the close sequence homology between PfHDAC1 and human class I HDACs<sup>21</sup> an

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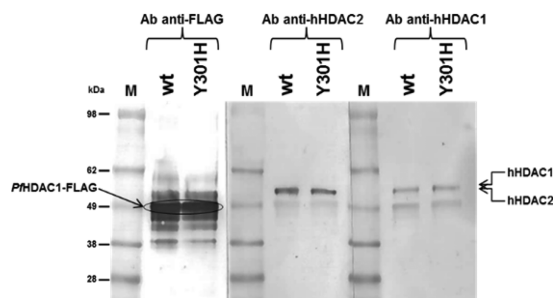
undesired effect of the use of HDAC inhibitors is potential toxicity resulting from induction of epigenetic changes in host mammalian cells. Approved cytotoxic anticancer agents such as Vorinostat operate via inhibition of human HDAC enzymes (*h*HDACs) and can cause mechanism based toxic side effects.<sup>22</sup> Thus, the development of selective inhibitors for *Pf*HDACs is viewed as necessary to circumvent potential toxicity issues in the host, and we report here our efforts in this direction. This work led to the development of the first selective series of inhibitors of *Pf*HDACs reported to date.

Previous research in our laboratories led to a novel series of heterocyclic inhibitors of human class-I HDACs represented by compound **3** (Figure 1).<sup>23</sup> This compound class features a central heterocycle that displays the pharmacophore elements common to many inhibitors of HDAC enzymes. Thus, a zinc binding group (ZBG) believed to interact with the catalytic metal ion of the HDAC enzyme is anchored by an alkyl linker. The ZBG is complemented by two surface contact groups (namely, the aryl substituent and the amide group) that are thought to interact close to the entrance of the substrate binding channel.<sup>24</sup> With many compounds from this structural class available, our efforts toward selective inhibitors of *Pf*HDACs logically began through screening a representative panel of human HDAC1 inhibitors against a recombinant *Pf*HDAC1 enzyme. The *Pf*HDAC1 enzyme required for this campaign was expressed in fusion with a flag protein tag, in either insect or mammalian cell lines, and was purified with an Anti-Flag affinity gel column.

In addition, *Pf*HDAC1 enzyme mutated in a Tyr residue (Y301H) located in the putative catalytic site was expressed. Mutation of the corresponding Tyr-to-His in human class-I HDACs has been demonstrated to severely reduce their enzymatic activities without affecting the overall protein structure, demonstrating that Y301 is essential for catalytic turnover.<sup>25</sup> Y301H *Pf*HDAC1 in fusion with a flag tag was expressed and purified under analogous conditions to the wild-type enzyme. Both purified proteins showed a low, though detectable, deacetylase activity in *in vitro* assays with fluorogenic substrates.

Compound **3** was tested head to head on mutant and wild type *Pf*HDAC1. No loss of enzyme activity nor inhibition was observed with the mutant enzyme, despite its (presumed) lack of catalytic efficiency. While it cannot be excluded that the role of Y301 in the catalytic mechanism of *Pf*HDAC1 differs from that observed for the corresponding residue in human class I HDACs, these results (together with the low *in vitro* activities observed across a variety of expression vectors in both mammalian and insect cell lines) added to our concern that the biological activities being measured were not in fact attributable to the *Pf*HDAC1 enzyme. This conclusion was ultimately supported by the finding that both *h*HDAC1 and *h*HDAC2 could be clearly detected by Western Blot using specific antihuman HDAC antibodies following the expression and purification of either mutant or wild-type *Pf*HDAC1 from human HeLa cells (Figure 2). The weight of evidence indicates that *Pf*HDAC1 is expressed as an inactive enzyme that likely needs endogenous cofactors for its biological activation, and consequently that biological results reported to date on inhibition of *Pf*HDAC1 are potentially false positives stemming from the presence of copurified host HDACs from the cellular expression vectors, likely associated in complex with *Pf*HDAC, at least in the expression systems used.

The apparent lack of catalytic activity following expression of *Pf*HDAC1 posed a significant challenge for the development of selective inhibitors of this enzyme. Although cell based assays



**Figure 2.** Western Blot analysis of purified wt and Y301H mutant *Pf*HDAC1s using anti-Flag, anti-*h*HDAC2, or anti-*h*HDAC1. Arrows indicate the position of the probed protein. M, molecular weight marker.

were available to measure either inhibition of *P. falciparum* growth in erythrocytes (*Pf*growth assay) or blockade of human class I HDACs in HeLa cells, exclusive use of the comparison of these two assays to determine reliable enzyme selectivities was viewed as precarious. Differences in cell type and assay protocol are likely to influence properties such as protein binding and cell penetration, and together with the inherently larger variability in cell-based assay data as compared to that from biochemical assays may significantly impair interpretations based on direct comparisons. Although *in vitro* assays measuring cell-penetration and protein binding might aid interpretation of results, issues with compound throughput under such a screening paradigm were viewed as problematic.

To alleviate the risks associated with our need to use a cell-based assay as a front-line screen for the development of selective inhibitors of *Pf*HDACs we have undertaken an approach outlined as follows: (i) comparison of parasite growth inhibition in erythrocytes with human class I HDAC inhibition in HeLa cells was used as a preliminary readout of selectivity with multiple repetitions of the cell based assays in order to minimize interpretation errors resulting from assay variability; (ii) structure–activity relationships were focused on improving parasite growth inhibition while *reducing* activity against the human HDAC1 enzyme; (iii) apparent selectivities measured in cell-based assays were validated at the biochemical level by measurement of nuclear histone hyperacetylation in both human and parasite treated cells. The inclusion of histone hyperacetylation experiments, as well as screening against the *h*HDAC1 enzyme amounts to an approach that adds weight to data obtained from simple comparison of cell-based assay data.

Screening results for a subset of our available compound collection in both the *P. falciparum* growth and human class I HDAC cellular assays highlighted an apparently low degree of selectivity, with most compounds inhibiting parasite growth at concentrations comparable with their human class I HDAC inhibition EC<sub>50</sub>. However, a group of outliers that featured significantly improved selectivity for parasite growth inhibition and shared a common structural feature was clearly evident. The bulk of compounds tested (e.g., compound **4**, Table 1) featured either ketone or hydroxamic acid zinc binding moieties, but the outliers in contrast were based upon a secondary amide as ZBG (e.g., compound **5**). This finding was somewhat surprising based on the conserved nature of the active site across different HDACs. An often asserted view is that selectivity between *P. falciparum* and human HDACs is likely to be realizable by modification of the surface contact groups in the pharmacophore, while our observation was that a change to the zinc binding group was impacting apparent selectivity.<sup>21,26,27</sup> In line

Table 1. SAR of Compounds 4–8<sup>a,18</sup>

cpd	Structure	<i>Pf</i>	HeLa		
		growth	class I	<i>h</i> HDAC1	SI <sup>c</sup>
		EC <sub>50</sub> <sup>b</sup>	IC <sub>50</sub> <sup>b</sup>	IC <sub>50</sub> <sup>b</sup>	
4		0.038 ± 0.008	0.094 ± 0.03	0.0014 ± 0.0004	2.5
5		0.31 ± 0.08	3.77 ± 1.04	0.34 ± 0.14	12
6		0.52 ± 0.03	2.45 ± 0.64	0.25 ± 0.08	4.7
7		0.57 ± 0.19	0.89 ± 0.30	0.20 ± 0.07	1.6
8		1.76 ± 0.36	6.65 ± 2.18	1.76 ± 0.38	3.8

<sup>a</sup>IC<sub>50</sub> and EC<sub>50</sub> values in  $\mu\text{M}$ . Compound 3 was used as standard reference compound (see data in Supplementary Table 1). <sup>b</sup>IC<sub>50</sub> and EC<sub>50</sub> values are the average of at least three individual measurements  $\pm$  SD. <sup>c</sup>SI: selectivity index (HeLa class I HDAC ( $\mu\text{M}$ )/*Pf*growth ( $\mu\text{M}$ )).

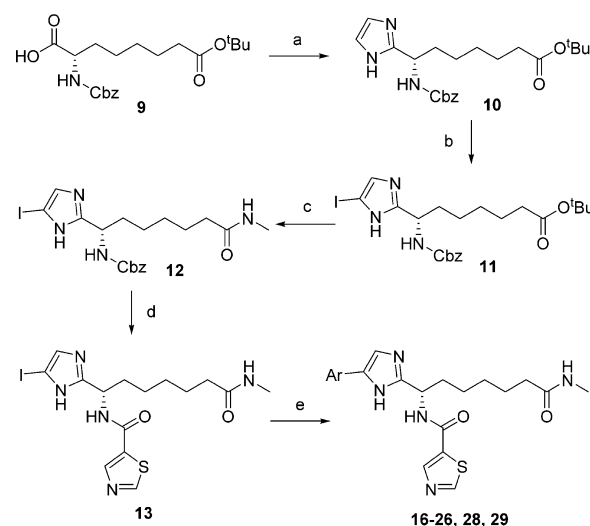
with our goal of optimizing away from potent human HDAC enzyme inhibitors an attractive feature was the much lower *h*HDAC1 enzyme inhibition shown by compound 5 that contains a methylamide as ZBG. Compound 5 is around an order of magnitude weaker than 4 in terms of parasite growth inhibition. However, in line with the lower *h*HDAC enzyme inhibition for the amide, a much stronger drop in cellular inhibition of human class I HDACs was noted, resulting in an overall improvement in the apparent selectivity. That the binding orientations of 4 and 5 are similar and that the methylamide (rather than the imidazole ring) in 5 is functioning as the ZBG are supported by several pieces of unpublished data. Notably, the imidazole ring in compound 5 can be replaced with alternative ring systems that are weak Zn<sup>2+</sup> binders without loss of parasite growth inhibition (data not shown).

In view of the expected high metabolic turnover for compounds containing a naphthyl ring as the aryl substituent,<sup>23</sup> the 2-methoxyquinoline analogue 6 was prepared.<sup>28</sup> This structural change (naphthyl to 2-methoxyquinoline) has previously been employed to favor reduced rodent clearance for human HDACis, and pharmacokinetic studies of 5 and 6 in mouse indeed demonstrated that plasma clearance was ameliorated for the latter compound (60 vs 10 mL/min/kg). However, neither of these analogues achieved detectable plasma exposure following oral administration, with portal vein sampling studies confirming that poor intestinal absorption was an issue for the methylamide ZBG analogues. Detectable oral exposure was, however, achievable by installing neutral functionality in place of the basic amine group present in 6. Thus, both compounds 7 and 8 showed a degree of oral bioavailability ( $F = 10\%$  and  $20\%$ , respectively) in mouse. The neutral amide analogues (7 and 8) showed weaker *P. falciparum* growth inhibition and/or failed to show any apparent selectivity, but taken as a whole, the overall characteristics of the methylamide

ZBG analogues in Table 1 showed potential as a route toward selective and potentially orally available inhibitors of *P. falciparum* growth.

Given the detrimental effect of basic functionality on oral absorption, the electro-neutral thiazole amide fragment was retained during efforts to align potency, selectivity, and oral absorption. In order to allow efficient exploration of SAR at the aryl substituent on the imidazole core a new synthetic strategy was developed based the introduction of the aryl moiety by Suzuki–Miyaura cross-coupling chemistry as the last step of the synthesis. Starting from the known amino acid 9 (Scheme 1),<sup>28</sup>

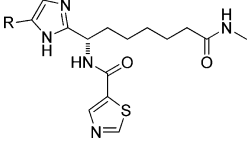
### Scheme 1. Chemistry for SAR at Aryl Substituent<sup>a</sup>

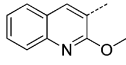
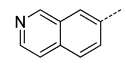
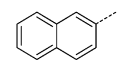
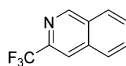
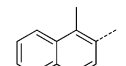
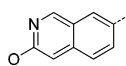
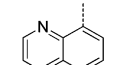
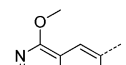
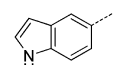
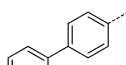
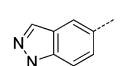
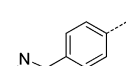
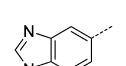
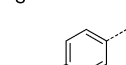
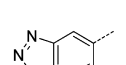
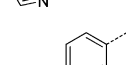
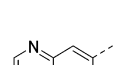
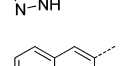


<sup>a</sup>Reagents and conditions: (a) (i) MeNHOMe.HCl, HBTU, DIPEA, DMF, RT; (ii) LiAlH<sub>4</sub>, THF,  $-20\text{ }^\circ\text{C}$ ; (iii) glyoxal, NH<sub>3</sub>, MeOH, RT; (b) (i) NIS, CH<sub>3</sub>CN,  $-10\text{ }^\circ\text{C}$ ; (ii) Na<sub>2</sub>SO<sub>3</sub>, Bu<sub>4</sub>NHSO<sub>4</sub>, dioxane–water,  $160\text{ }^\circ\text{C}$ , microwave, 30 min; (c) (i) TFA, DCM, RT; (ii) MeNH<sub>2</sub>, HBTU, DMF, RT; (d) (i) HBr in AcOH, DCM,  $0\text{ }^\circ\text{C}$  to RT; (ii) thiazole-2-carboxylic acid, EDC.HCl, HOBt, DIPEA, DMF, RT; (e) ArB(OH)<sub>2</sub>, PdCl<sub>2</sub>(dppf), K<sub>2</sub>CO<sub>3</sub>, DME–water,  $110\text{ }^\circ\text{C}$ .

the free carboxylic acid was transformed into an imidazole ring in three synthetic steps.<sup>29</sup> Iodination of the imidazole was accomplished by employing a halogenation/dehalogenation protocol that furnished the desired monoiodinated compound 11. The methyl amide ZBG was installed by ester deprotection and subsequent coupling with methylamine to give the synthetically versatile intermediate 12. Removal of the Cbz protecting group in this compound and installation of the thiazole amide furnished the substrate for final Suzuki reactions. Cross couplings between 13 and boronic acids (or esters) proceeded smoothly when PdCl<sub>2</sub> was employed as a precatalyst,<sup>30</sup> furnishing (after HPLC purification) compounds 16–26, 28, and 29 (Scheme 1). Compounds 14,<sup>28</sup> 15, 27, and 30 were synthesized using alternative procedures that are described in the Supporting Information.

The results in Table 2 highlight that both *Plasmodium* growth potency as well as selectivity with respect to inhibition of human class I HDACs in HeLa cells were strongly influenced by the nature of the aryl substituent attached to the imidazole core. Although clear SAR trends were difficult to identify, there were indications that the aryl group attached to the imidazole ring is involved in a specific interaction with *Pf*HDAC. Similar to the trend observed between compounds 5 and 6, the 2-naphthyl compound 14 was a marginally more potent and selective

Table 2. SAR of Compounds 7 and 14–30<sup>a</sup>


cpd	R	<i>Pf</i>	HeLa	<i>h</i> HDAC1		cpd	R	<i>Pf</i>	HeLa	<i>h</i> HDAC1	
		growth	class I	IC <sub>50</sub> <sup>b</sup>	SI <sup>c</sup>			growth	class I	IC <sub>50</sub> <sup>b</sup>	SI <sup>c</sup>
		EC <sub>50</sub> <sup>b</sup>	HDAC	IC <sub>50</sub> <sup>b</sup>				EC <sub>50</sub> <sup>b</sup>	HDAC	IC <sub>50</sub> <sup>b</sup>	
7		0.57 ± 0.19	0.89 ± 0.30	0.20 ± 0.07	1.6	22		0.27 ± 0.12	4.75 ± 1.44	0.60 ± 0.15	18
14		0.30 ± 0.12	1.70 ± 0.57	0.32 ± 0.08	5.7	23		0.10 ± 0.02	2.04 ± 0.71	0.29 ± 0.04	20
15		23.73 ± 1.50	> 25	23.94 ± 0.89	1	24		0.10 ± 0.05	1.37 ± 0.38	0.39 ± 0.02	14
16		8.28 ± 1.59	> 25	> 2.5	3	25		4.86 ± 3.09	20.14 ± 2.91	2.62 ± 1.57	4.1
17		0.33 ± 0.10	6.33 ± 1.42	1.28 ± 0.22	19	26		7.57 ± 0.95	> 25	> 5	3.3
18		0.63 ± 0.09	7.63 ± 0.50	2.12 ± 0.35	12	27		0.57 ± 0.07	8.13 ± 0.01	2.16 ± 0.30	14
19		12.04 ± 1.70	> 25	> 2.5	2.1	28		0.90 ± 0.23	20.49 ± 0.43	2.49 ± 1.02	23
20		10.05 ± 5.39	> 25	> 5	2.5	29		0.45 ± 0.08	16.63 ± 3.26	1.85 ± 0.31	37
21		3.81 ± 0.50	> 25	> 5	6.6	30		0.5 ± 0.17	> 25	> 2.5	54

<sup>a</sup>IC<sub>50</sub> and EC<sub>50</sub> values in  $\mu\text{M}$ . Compound 3 was used as standard reference compound (see data in Supplementary Table 1). <sup>b</sup>IC<sub>50</sub> and EC<sub>50</sub> values are the average of at least three individual measurements  $\pm$  SD. <sup>c</sup>SI: selectivity index (HeLa class I HDAC( $\mu\text{M}$ )/*Pf*growth( $\mu\text{M}$ )).

inhibitor of *Plasmodium* growth than 7. However, introduction of a methyl group at position 1 of the naphthyl ring (compound 15) completely abolished *P. falciparum* growth inhibition. Such a strong influence on activity resulting from introduction of a single methyl group likely points to a change in enzyme inhibition rather than a physicochemical effect that impacts cell-based activity. Similarly, while many 3,4-fused bicyclic heterocycles generated submicromolar *P. falciparum* growth inhibition (compounds 5–7, 14) no active compounds based on a 2,3-fused bicyclic ring system were found throughout the course of the program. The weak micromolar growth inhibition shown by compound 16 was illustrative of results typically obtained for 2,3-fused heterocycles. A wide variety of 3,4-fused bicyclic heterocycles were evaluated, and compounds 17–20 highlight the somewhat empirical nature of this exploration. The indole and indazole analogues (17 and 18, respectively) retained submicromolar growth inhibition and showed 10-fold improved selectivity with respect to the 2-methoxyquinoline analogue 7

that was our starting point. Furthermore, these compounds provided a first indication that *P. falciparum* growth inhibition in erythrocytes is stronger than activity measured in the human HDAC1 biochemical assay. In contrast, the benzimidazole analogue 19 and the benzotriazole analogue 20 were weak and poorly selective inhibitors of *Plasmodium* growth. Throughout the program we were unable to correlate trends between activities and measured or calculated physicochemical properties (e.g., logD) that may account for such differences in profile. Very different results were again obtained within a set of isomeric quinoline/isoquinoline analogues. Of the five isomers that were prepared, quinoline analogue 21 proved to be a very weak inhibitor, while the 4-isoquinoline 22 emerged as the most promising. Compound 22 showed submicromolar *Plasmodium* growth inhibition and 18-fold selectivity with respect to the human HDAC cellular assay. Substituted analogues of 22 were explored, with 23 and 24 both showing improved growth inhibition in the 100 nM range and comparable selectivity. In line

with a specific enzyme interaction for the aryl group as previously described, the positioning of the quinoline substituent was crucial, with the 3-substituted analogue **25** showing weak *P. falciparum* growth inhibitory activity. Compounds **23** and **24** represent the most potent inhibitors that were prepared, but further improvements in selectivity with respect to human cellular HDAC inhibition were achieved.

Very weak inhibition of *Plasmodium* growth was measured for numerous phenyl-substituted imidazoles, including the biphenyl analogue **26**, which is a 7  $\mu\text{M}$  inhibitor. However, 6,5-biaryl systems turned out to show improved activity with the 4-(2,4)-thiazole analogue **27** and 4-(1,2)-pyrazole analogue **28** both showing submicromolar *P. falciparum* growth inhibition and selectivities around 15–20-fold. A further improvement in selectivity was achieved with the isomeric pyrazole **29**, which was almost 40-fold selective for parasite growth. The most selective compound overall was the 2-hydroxy-3-quinoline analogue **30**, which in multiple tests consistently failed to show activity in our human HDAC assays. The weak activity of **30** against human cellular class I HDACs meant that no  $\text{IC}_{50}$  value was determined; the apparent selectivity of the compound is estimated as being greater than 50-fold (based on the upper concentration at which it was tested in the assay). Notably, compounds **29** and **30** have *hHDAC1* enzyme inhibition 4–5-fold weaker than activity in cell-based *Plasmodium* growth assay, supporting the notion that these are selective inhibitors of *PfHDACs* and in line with our goal of optimizing away from strong *hHDAC1* enzyme inhibition.

Due to the lack of a reliable biochemical assay in which to measure enzymatic *PfHDAC* inhibition directly, the mechanism of action by which the optimized inhibitors elicited their antiparasitic activity was studied by histone hyperacetylation experiments. Assuming the inhibitors block the histone deacetylation process in parasites, then a phenotype that shows hyperacetylation of parasite histone proteins would be expected. Treatment of *P. falciparum* parasites with increasing concentrations of **29**, one our most potent and selective inhibitors, and analysis of histone H4 lysine 8 clearly confirmed hyperacetylation at a concentration ( $\text{EC}_{50} = 350 \text{ nM}$ ) close to the compounds  $\text{EC}_{50}$  measured in the parasite growth assay ( $\text{EC}_{50} = 450 \text{ nM}$ , Supplementary Figure 2 in the Supporting Information section). Furthermore, little histone hyperacetylation of histone H4 was observed at concentrations up to 25  $\mu\text{M}$  when human HeLa cells were treated with **29**, in agreement with the HDAC activity measured in this cell line ( $\text{EC}_{50} = 16.7 \mu\text{M}$ ). These results, which were reproduced with several additional compounds (data not shown), support *P. falciparum* HDAC inhibition as the mechanism of action and also appear to corroborate the idea that comparison of *P. falciparum* growth inhibition and human cellular class I HDAC inhibition data can provide meaningful insight with regard to biochemical events within the cells. Further profiling of compound **29** against a panel of human HDAC enzymes (*hHDAC* 1–7, 9) showed that for most isoenzymes no inhibition was observed up to 5  $\mu\text{M}$ , though *hHDAC3* was inhibited with  $\text{IC}_{50} = 330 \text{ nM}$ . No significant toxicity was observed for **29** in HeLa or HUVEC cells ( $\text{CC}_{50} > 25,000 \text{ nM}$ ) nor did the compound bind to *hERG* ion channels ( $\text{IC}_{50} > 30,000 \text{ nM}$ ). While **29** showed moderate intrinsic clearances in rat, human, and mouse liver microsomes ( $\text{Cl}_{\text{int}} = 27, 19, \text{ and } 12 \mu\text{L}/\text{min}/\text{mgP}$ , respectively), it had clearance above liver blood flow following *i.v.* administration in mouse ( $\text{Cl}_{\text{p}} = 247 \text{ mL}/\text{min}/\text{kg}$ ). Efforts to understand the apparent extra-hepatic clearance, which does not appear to stem from instability in mouse plasma,

and combine potency and selectivity with oral exposure remain under investigation in our laboratories.

In summary, we have reported findings that cast doubt on *PfHDAC1* enzyme inhibition data reported to date in the literature<sup>31</sup> and have described a screening approach that guided the discovery of what we believe to be selective inhibitors of *Plasmodium falciparum* HDACs. The leading compounds from this work show submicromolar inhibition of *P. falciparum* growth ( $\text{EC}_{50} < 500 \text{ nM}$ ), have good selectivity (>50-fold) over human HDACs in cells, and are weak inhibitors of human HDAC enzymes. Support for the mechanism of action of this compound class was provided by histone hyperacetylation studies.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.5b00468.

Synthetic experimental details and characterization data, description of primary biological assay protocols, and PK protocols (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

DNA, deoxyribonucleic acid; HDAC, histone deacetylase; ZBG, zinc-binding group; SAR, structure–activity relationship; *hERG*, human Ether-a-go-go-Related Gene; HUVEC, human umbilical vein endothelial cell; Cbz, carboxybenzyl; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIPEA, *N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; EDC, *N*-ethyl-*N'*-(3-(dimethylamino)propyl)-carbodiimide; HPLC, high performance liquid chromatography; PdCl<sub>2</sub>(dppf), [1,1'-Bis(diphenylphosphino)ferrocene]-dichloropalladium(II)

## REFERENCES

- (1) WHO. *World Malaria Report 2014*; World Health Organization: Geneva, 2014.
- (2) Turschner, S.; Efferth, T. Drug resistance in Plasmodium: Natural products in the fight against malaria. *Mini-Rev. Med. Chem.* **2009**, *9*, 206–214.
- (3) Hyde, J. E. Drug-resistant malaria – an insight. *FEBS J.* **2007**, *274*, 4688–4698.
- (4) Philips, D. M. The presence of acetyl groups of histones. *Biochem. J.* **1963**, *87*, 258–263.
- (5) Allfrey, V. G.; Faulkner, R.; Mirsky, A. E. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of RNA Synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **1964**, *51*, 786–794.
- (6) Gershey, E. L.; Vidali, G.; Allfrey, V. G. Chemical studies of histone acetylation. The occurrence of epsilon-N-acetyllysine in the f2a1 histone. *J. Biol. Chem.* **1968**, *243*, 5018–5022.
- (7) Chaal, B. K.; Gupta, A. P.; Wastuwidyaningtyas, B. D.; Luah, Y. H.; Bozdech, Z. Histone deacetylases play a major role in the transcriptional regulation of the *Plasmodium falciparum* life cycle. *PLoS Pathog.* **2010**, *6*, e1000737.
- (8) Duraisingh, M. T.; Voss, T. S.; Marty, A. J.; Duffy, M. F.; Good, R. T.; Thompson, J. K.; Freitas-Junior, L. H.; Scherf, A.; Crabb, B. S.; Cowman, A. F. Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in *Plasmodium falciparum*. *Cell* **2005**, *121*, 13–24.
- (9) Freitas-Junior, L. H.; Hernandez-Rivas, R.; Ralph, S. A.; Montiel-Condado, D.; Ruvalcaba-Salazar, O. K.; Rojas-Meza, A. P.; Mancio-Silva, L.; Leal-Silvestre, R. J.; Marques Gontijo, A.; Shorte, S.; Scherf, A. Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **2005**, *121*, 25–36.
- (10) Hu, G.; Cabrera, A.; Kono, M.; Mok, S.; Chaal, B. K.; Haase, S.; Engelberg, K.; Cheemadan, S.; Spielmann, T.; Preiser, P. R.; Gillberger, T.-W.; Bozdech, Z. Transcriptional profiling of growth perturbations of the human malaria parasite *Plasmodium falciparum*. *Nat. Biotechnol.* **2010**, *28*, 91–98.
- (11) Dow, G. S.; Chen, Y.; Andrews, K. T.; Caridha, D.; Genera, L.; Gettayacamin, M.; Johnson, J.; Li, Q.; Melendez, V.; Obaldia, N., III; Tran, T. N.; Kozikowski, A. P. Antimalarial activity of phenylthiazolyl-bearing hydroxamate-based histone deacetylase inhibitors. *Antimicrob. Agents Chemother.* **2008**, *52*, 3467–3477.
- (12) Marfurt, J.; Chalfein, F.; Prayoga, P.; Wabiser, F.; Kenangalem, E.; Piera, K. A.; Fairlie, D. P.; Tjitra, E.; Anstey, N. M.; Andrews, K. T.; Price, R. N. *Ex vivo* activity of histone deacetylase inhibitors against multidrug-resistant clinical isolates of *Plasmodium falciparum* and *P. vivax*. *Antimicrob. Agents Chemother.* **2011**, *55*, 961–966.
- (13) Hansen, F. K.; Sumanadasa, S. D. M.; Stenzel, K.; Duffy, S.; Meister, S.; Marek, L.; Schmetter, R.; Kuna, K.; Hamacher, A.; Mordmuller, B.; Kassak, M. U.; Winzeler, E. A.; Avery, V. M.; Andrews, K. T.; Kurz, T. Discovery of HDAC inhibitors with potent activity against multiple malaria parasite life cycle stages. *Eur. J. Med. Chem.* **2014**, *82*, 204–213.
- (14) Hansen, F. K.; Skinner-Adams, T. S.; Duffy, S.; Marek, L.; Sumanadasa, S. D. M.; Kuna, K.; Held, J.; Avery, V. M.; Andrews, K. T.; Kurz, T. Synthesis, Antimalarial Properties, and SAR Studies of Alkoxyurea-Based HDAC Inhibitors. *ChemMedChem* **2014**, *9*, 665–670.
- (15) Giannini, G.; Battistuzzi, G.; Vignola, D. Hydroxamic Acid Based Histone Deacetylase Inhibitors with Confirmed Activity against the Malaria Parasite. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 459–461.
- (16) Engel, J. A.; Jones, A. J.; Avery, V. M.; Sumanadasa, S. D. M.; Ng, S. S.; Fairlie, D. P.; Adams, T. S.; Andrews, K. T. Profiling the anti-protozoal activity of anti-cancer HDAC inhibitors against Plasmodium and Trypanosoma parasites. *Int. J. Parasitol.: Drugs Drug Resist.* **2015**, *5*, 117–126.
- (17) Sumanadasa, S. D. M.; Goodman, C. D.; Lucke, A. J.; Skinner-Adams, T.; Sahama, I.; Haque, A.; Do, T. A.; McFadden, G. I.; Fairlie, D. P.; Andrews, K. T. Potent Antimalarial Activity of Histone Deacetylase Inhibitor Analogues. *Antimicrob. Agents Chemother.* **2012**, *56*, 3849–3856.
- (18) Andrews, K. T.; Haque, A.; Jones, M. K. HDAC Inhibitors in Parasitic Diseases. *Immunol. Cell Biol.* **2012**, *90*, 66–77.
- (19) Andrews, K. T.; Tran, T. N.; Wheatley, N. C.; Fairlie, D. P. Targeting Histone Deacetylase Inhibitors for Anti-Malarial Therapy. *Curr. Top. Med. Chem.* **2009**, *9*, 292–308.
- (20) Trenholme, K.; Marek, L.; Duffy, S.; Pradel, G.; Fisher, G.; Hansen, F. K.; Skinner-Adams, T. S.; Butterworth, A.; Ngwa, C. J.; Moecking, J.; Goodman, C. D.; McFadden, G. I.; Sumanadasa, S. D. M.; Fairlie, D. P.; Avery, V. M.; Kurz, T.; Andrews, K. T. Lysine Acetylation in Sexual Stage Malaria Parasites Is a Target for Antimalarial Small Molecules. *Antimicrob. Agents Chemother.* **2014**, *58*, 3666–3678.
- (21) Mukherjee, P.; Praddhan, A.; Shah, F.; Tekwani, B. L.; Avery, M. A. Structural insights into the *Plasmodium falciparum* histone deacetylase 1 (PfHDAC-1): A novel target for the development of antimalarial therapy. *Bioorg. Med. Chem.* **2008**, *16*, 5254–5265.
- (22) Mann, B. S.; Johnson, J. R.; Cohen, M. H.; Justice, R.; Pazdur, R. FDA Approval summary: Vorinostat for Treatment of Advanced Primary Cutaneous T-Cell Lymphoma. *Oncologist* **2007**, *12*, 1247–1252.
- (23) Kinzel, O.; Llauger-Bufi, L.; Pescatore, G.; Rowley, M.; Schultz-Fademrecht, C.; Monteagudo, E.; Fonsi, M.; Gonzalez Paz, O.; Fiore, F.; Steinkühler, C.; Jones, P. Discovery of Potent Class I Selective Ketone Histone Deacetylase Inhibitor with Antitumor Activity in Vivo and Optimized Pharmacokinetic Properties. *J. Med. Chem.* **2009**, *52*, 3453–3456.
- (24) Vannini, A.; Volpari, C.; Gallinari, P.; Jones, P.; Mattu, M.; Carfi, A.; De Francesco, R.; Steinkühler, C.; Di Marco, S. Substrate binding to histone deacetylases as shown by the crystal structure of the HDAC8-substrate complex. *EMBO Rep.* **2007**, *8*, 879–884.
- (25) Lahm, A.; Paolini, C.; Pallaoro, M.; Nardi, M. C.; Jones, P.; Neddermann, P.; Sambucini, S.; Bottomley, M. J.; Lo Surdo, P.; Carfi, A.; Koch, U.; De Francesco, R.; Steinkühler, C.; Gallinari, P. Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 17335–17340.
- (26) Agbor-Enoh, S.; Seudieu, C.; Davidson, E.; Dritschilo, A.; Jung, M. Novel Inhibitor of plasmodium Histone Deacetylase That cures P. berghei-Infected Mice. *Antimicrob. Agents Chemother.* **2009**, *53*, 1727–1734.
- (27) Andrews, K. T.; Tran, T. N.; Lucke, A. J.; Kahnberg, P.; Le, G. T.; Boyle, G. M.; Gardiner, D. L.; Skinner-Adams, T. S.; Fairlie, D. P. Potent Antimalarial Activity of Histone Deacetylase Inhibitor Analogues. *Antimicrob. Agents Chemother.* **2008**, *52*, 1454–1461.
- (28) Compounds **4–9** and **14** were synthesized using the procedures described in Atenni, B.; Ferrigno, F.; Jones, P.; Ingenito, R.; Kinzel, O.; Llauger-Bufi, L.; Ontoria, J. M.; Pescatore, G.; Rowley, M.; Scarpelli, R.; Schultz-Fademrecht, C. Heterocycles Derivatives as Histone Deacetylase (HDAC) Inhibitors. WIPO Patent 061638, 2006.
- (29) Deziel, R.; Rahil, J.; Wahhab, A.; Allan, M.; Nguyen, N. Sirtuin Inhibitors. WIPO Patent 026701, 2009.
- (30) Zeng, Q.; Chen, K. X.; Anilkumar, G. N.; Rosenblum, S. B.; Kozłowski, J. A.; Njoroge, F. G. Antiviral Compounds Composed of Three Linked Aryl Moieties to Treat Diseases such as Hepatitis C. WIPO Patent 138791, 2010.
- (31) Patel, V.; Mazitschek, R.; Coleman, B.; Nguyen, C.; Urgaonkar, S.; Cortese, J.; Barker, R. H., Jr.; Greenberg, E.; Tang, W.; Bradner, J. E.; Schreiber, S. L.; Duraisingh, M. T.; Wirth, D. F.; Clardy, J. Identification and Characterization of Small Molecule Inhibitors of a Class I Histone Deacetylase from *Plasmodium falciparum*. *J. Med. Chem.* **2009**, *52*, 2185–2187.