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Antimalarial histone deacetylase inhibitors containing cinnamate or NSAID components

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ABSTRACT

Malaria is the most lethal parasite-mediated tropical infectious disease, killing 1–2 million people each year. An emerging drug target is the enzyme *Plasmodium falciparum* histone deacetylase 1 (PfHDAC1). We report 26 compounds designed to bind the zinc and exterior surface around the entrance to the active site of PfHDAC1, 16 displaying potent in vitro antimalarial activity (IC_{50} <100 nM) against *P. falciparum*. Selected compounds were shown to cause hyperacetylation of *P. falciparum* histones and be >10-fold more cytotoxic towards *P. falciparum* than a normal human cell type (NFF). Twenty-two inhibitors feature cinnamic acid derivatives or non-steroidal anti-inflammatory drugs (NSAIDs) as HDAC-binding components. A homology model of PfHDAC1 enzyme gives new insights to interactions likely made by some of these inhibitors. Results support PfHDAC1 as a promising new antimalarial drug target.

Malaria is a mosquito-borne infectious disease caused by protozoan parasites. It is widespread in tropical and subtropical regions and each year there are approximately 500 million clinical cases of malaria. An estimated 1–2 million people die each year of malaria related disease and most are children under the age of five.[1] There is currently no licensed malaria vaccine available. Effective treatment and control of malaria continues to be challenged by parasite resistance to antimalarial drugs,[2,3] prompting renewed efforts to discover compounds with new mechanisms of action on novel targets in the parasite.

Histone deacetylase enzymes in malaria parasites may represent potential new targets for antimalarial drug development. HDACs are Zn-dependent enzymes that play crucial roles in modulating mammalian cell chromatin structure, transcription, and gene expression.[4,5] HDAC inhibitors can arrest growth and induce differentiation and/or apoptotic cell death in various human cancer cells lines[6,7] and some HDAC inhibitors also possess antimalarial activity.[8–12] Compounds like apicidin **1**, TSA **2**, SAHA **3**, and compound **4** derived from L-2-aminosuberic acid (Asu) (Fig. 1), inhibit HDAC enzymes by binding in the zinc-containing active site.[9,13]

Plasmodium falciparum contains at least five putative HDAC enzymes as well as homologs to other histone modifying proteins such as acetylase enzymes, although little is known about them.[14,15]

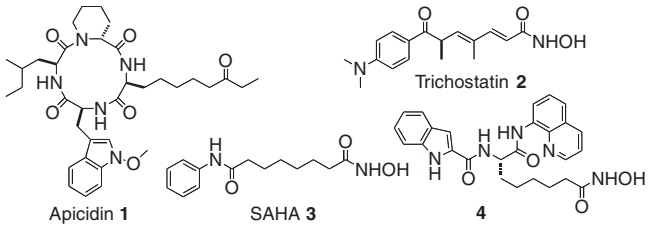

Figure 1. Examples of HDAC inhibitors with antimalarial activity.

Apicidin is known to cause dramatic transcriptional changes at all stages of the *P. falciparum* intraerythrocytic developmental cycle.[16] Of five known *P. falciparum* HDACs (PfHDACs), PfHDAC1 was found localized mainly in the parasite nucleus,[15] with sequence homology to human HDAC1, and is inhibited by **2** and **3**.[15,17] Homology models[9,18] of PfHDAC1 have been compared to crystal structures of human HDAC8. Subtle differences in amino acids that line the binding pocket, especially the exterior surface at the entrance to the tubular zinc-bound active site, might be exploited to develop selective PfHDAC1 inhibitors.

We have found that compounds derived from Asu, like **4** and **Asu-9**, have potent antimalarial activity[9] and also inhibit *P. falciparum* HDAC nuclear extracts in a dose-dependent manner (IC_{50} 78 nM and 87 nM, respectively, unpublished results). **Asu-9** was a lead compound with a cinnamic acid component. To

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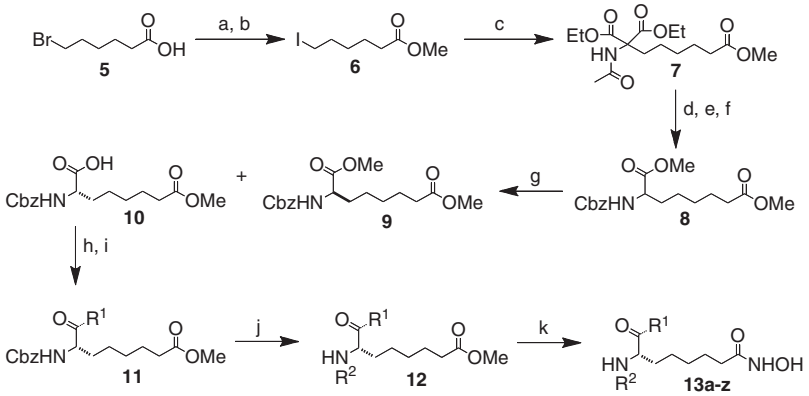


Scheme 1. Synthesis and resolution of antimalarial compounds. Reagents and conditions: (a) MeOH, cat. H_2SO_4, Δ; (b) acetone, NaI, Δ; (c) DMF, NaH, diethyl acetamidomalonate, rt; (d) HCl/H_2O, Δ; (e) MeOH, HCl, Δ; (f) EtOAc, Cbz-OSu, pH 8, rt; (g) DMF, 0.2 M NaOAc, Cys. HCl, *Carica Papaya*; (h) DMF, BOP, DIPEA, R^1 (amine) (16), rt; (i) MeOH/EtOAc, H_2, 10% Pd/C, cat. TFA, rt; (j) DMF, BOP, DIPEA, R^2 (acid), rt; (k) MeOH, $KO(CH_3)_3$, hydroxylamine hydrochloride, rt.

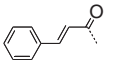
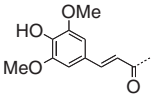
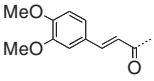
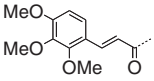
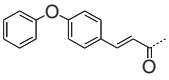
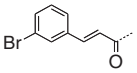
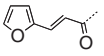
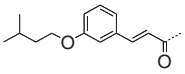
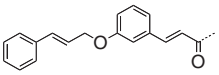
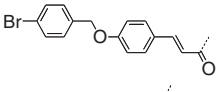
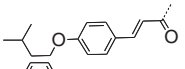
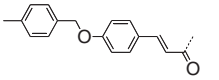
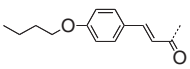
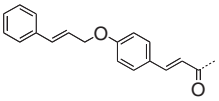
explore the active-site surface of PfHDAC1, a series of synthetically accessible derivatives of cinnamic acids, non-steroidal anti-inflammatory drugs (NSAIDs), and amines were synthesized. Inhibitor synthesis was performed according to Scheme 1. Compound **5** was protected as a methyl ester followed by iodide substitution forming **6**. Alkylation of diethyl acetamidomalonate with **6** gave **7**, which was hydrolyzed under acidic conditions followed by protection of the acid as the methyl ester, and conversion of the amine to the benzylcarbamate **8**. Chiral resolution of **8** was achieved using the cysteine protease, *Carica papaya*[19,20] and stopped at ~40% conversion to free acid **10** and ester **9**. Optical purity of **10** was determined by derivatizing the amino acid with a chiral auxiliary and examining the dipeptide with NMR experiments (Supplementary data). Functionalizing side chain groups R^1 and R^2 (Scheme 1) using standard coupling procedures gave a series of inhibitors.

Synthetic compounds had *C* log *P* values between 1.5 and 5.5 and were thus expected to be cell permeable. In vitro *P. falciparum* growth inhibition assays were performed as described.[9] Initially, cinnamic acid derivatives (**13a**–**m**, Table 1) were synthesized as putative HDAC inhibitors. Smaller compounds like **13a**–**c** with hydroxy/methoxy substituents on the cinnamyl aromatic ring were potent inhibitors (IC_{50} 14, 34, 52 nM, respectively), as were derivatives with *m*-bromo (**13e**, IC_{50} 50 nM) or furylacryl (**13f**, IC_{50} 15 nM) substituents. Cinnamic acid derivatives **13g**–**m** Table 1, with longer extended meta/para substituents showed ⩾10-fold decreased killing potency.

Since extended cinnamates were less potent, we decided to synthesize more compact rigid compounds. A series of conveniently available carboxylic acids were NSAIDs that were readily coupled to 8-aminoquinoline derived compounds (NSAIDs: Flubiprofen, Naproxen, Mefenamic acid, Diclofenac, Indomethacin, Aspirin, Tolmetin, Sulindac, and Indoprofen compounds; **13n**–**u**, Table 2, respectively). Partial hydrolysis of indomethacin and aspirin derivatives occurred, producing **13r** and **13s**, respectively. All but one of the NSAID derivatives had potent antimalarial activity (IC_{50} ⩽65 nM), none being too bulky or too elongated to prevent parasite killing.

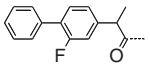
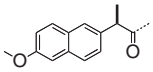
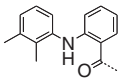
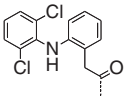
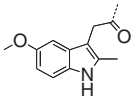
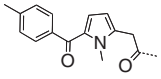
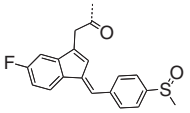
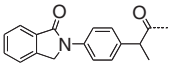
Previously, we reported **Asu-13** as a potent antimalarial compound.[9] To explore active-site space occupied by the 8-aminoquinoline group, four *p*-dimethylaminobenzoic acid substituted inhibitors were synthesized with differing amines (**13w**–**z**, Table 3). Compounds **13w**–**z** were all potent inhibitors (IC_{50} ⩽102 nM), but **13z** was the most potent and may be a valuable lead. Compounds **13o**, **13r**, **Asu-13** were also ⩾10 times more cytoselective for

Table 1
Antimalarial activity of cinnamic acid derived-inhibitors **13a**–**m** (R^1 = 8-aminoquinoline)

No.	R^2	*C* log *P*	IC_{50}[a] (nM)	IC_{90}[a] (nM)
Asu-9		2.9	15	nd
13a		1.8	14	26
13b		2.5	34	61
13c		2.2	52[b]	nd
13d		4.9	156[b]	nd
13e		3.7	50[b]	nd
13f		2.0	15	27
13g		4.8	>1000	>1000
13h		5.2	400	805
13i		5.4	357	687
13j		4.8	217	536
13k		5.0	153	375
13l		4.4	184	373
13m		5.2	453	827

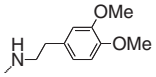
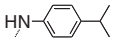
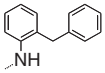
[a] Mean values *n* = 3.
[b] Duplicate experiments, nd = not determined.

Table 2
Antimalarial activity of compounds **13n–v** with NSAIDs as HDAC-binding components (R^1 = 8-aminoquinoline)

No.	R^2	*C* log *P*	IC_{50} [a] (nM)	IC_{90} [a] (nM)
13n		4.4	42	76
13o		3.4	14	24
13p		5.1	51	89
13q		5.3	65	119
13r		2.5	13	22
13s		2.6	115	214
13t		2.8	47	102
13u		3.7	35	35
13v		3.4	32	65

[a] Mean values $n = 3$.

Table 3
Antimalarial activity amine-derived inhibitors (R^2 = *p*-dimethylaminobenzoic acid)

No.	R^1	*C* log *P*	IC_{50} [a] (nM)	IC_{90} [a] (nM)
Asu-13		2.3	19	nd
13w		1.7	101	nd
13x		3.3	22	nd
13y		3.0	21	nd
13z		3.0	5	nd

[a] Mean values $n = 3$, nd = not determined.

Table 4
Inhibitors compared against *Pf*-3D7 and NFF cells

No.	3D7 IC_{50} [a] (nM)	NFFs IC_{50} [a] (nM)	SI [b]
13o	14	169	12
13r	13	260	20
Asu-13	19	570	30

[a] Mean of three experiments.
[b] Selectivity index = NFF IC_{50}/3D7 IC_{50}.

P. falciparum malaria parasites versus human normal neonatal foreskin fibroblast (NFF) cells (Table 4). Selectivity is variable depending on cell lines used,[9,12] but inhibitors with SI as low as 17 (3D7 vs HeLa cells) have been used to cure *Plasmodium berghei*-infected mice.[12]

To confirm that compounds investigated here act as HDAC inhibitors in malaria parasites, three representative compounds (**13o**, **13r**, and **13z**) were assessed for their capacity to hyperacetylate *P. falciparum* histones. All three compounds and TSA (as control) caused hyperacetylation of histone H4 in 3D7 parasites (Fig. 2), as determined by Western blot analysis using a commercial polyclonal anti-tetra-acetyl histone H4 antibody (Upstate). Artemisinin, a control antimalarial compound, did not affect histone H4 acetylation (Fig. 2).

A homology model of the PfHDAC1 sequence (PFI1260c) was created from crystal structures of a bacterial homolog HDLP (1C3R.pdb, 2.00 Å resolution)[21] for loop1, and human HDAC8 (using 2v5x.pdb, 2.25 Å resolution,[22] rather than multiple crystal structures used previously[9]) for the remainder of the protein (sequence identities 32% and 42%, respectively). The PfHDAC1 homology model has the same fold as Class 1 HDACs, consisting of a single α/β domain. β-Sheets form an interior scaffold of the enzyme and help support active-site residues surrounding the catalytic Zn. Active-site tunnel residues are identical to human Class I HDACs with loop residues near the entrance showing some variability. As the homology model was based on HDAC8, it is more flexible around loops 1 and 2 than PfHDAC1. Four pockets were identified near the active-site entrance (A–D Fig. 3a). GOLD was used to dock ligands in the active site using distance and H-bond constraints (Supplementary data).

Top ranked GOLD scoring conformations provided insights to possible binding orientations of **13a–z** (see Fig. 3). The HDAC8 crystal structure shows an Asu derived inhibitor 2V5X and a peptide interacting with HDAC8 via both backbone amide–NHs H-bonding in a *cis*-conformation to Asp97. GOLD docked smaller cinnamic acid inhibitors **13a–h**, **k**, and **l** with one or both (*cis*-conformation) Asu amide–NHs H-bonding to Asp97, the 8-aminoquinoline group placed into pocket C, and the cinnamate group in pocket A (inhibitors **13a**, **13d**; Fig. 3b). Other cinnamic acid derivatives were more extended with one or no H-bonds to Asp97, forcing side chains to access pockets B, C, and D (e.g., **13h** and **13i**, Fig. 3b).

Docked conformations of NSAID-derived inhibitors **13n–v** clearly showed the importance of the inhibitor backbone amide–NHs H-bonding to Asp97 with the 8-aminoquinoline group docked into pockets C and D with the NSAID groups binding in A or D (inhibitors **13n** yellow, **13p** orange, **13r** lt blue, Fig. 3c). The C-terminal derivatives **13w–z** docked with the *p*-dimethylaminobenzoic acid group binding in pocket A and the amine groups in

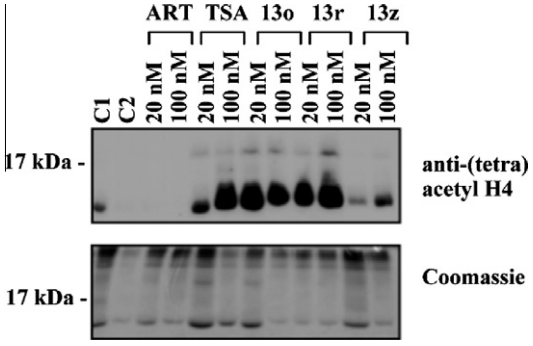


Figure 2. *P. falciparum* infected erythrocytes (3D7) were cultured in the presence of 20 and 100 nM artemisinin (ART), trichostatin A (TSA), **13o**, **13r**, or **13z** for 3.5 h. Matched controls taken at the start (C1) and at 3.5 h (C2) received vehicle alone (0.1% DMSO). Total protein was extracted and separated via 15% SDS–PAGE. Hyperacetylation was determined by Western blot using polyclonal anti-tetraacetyl histone H4 antisera (Upstate). Coomassie staining of a matched control gel showed loading.

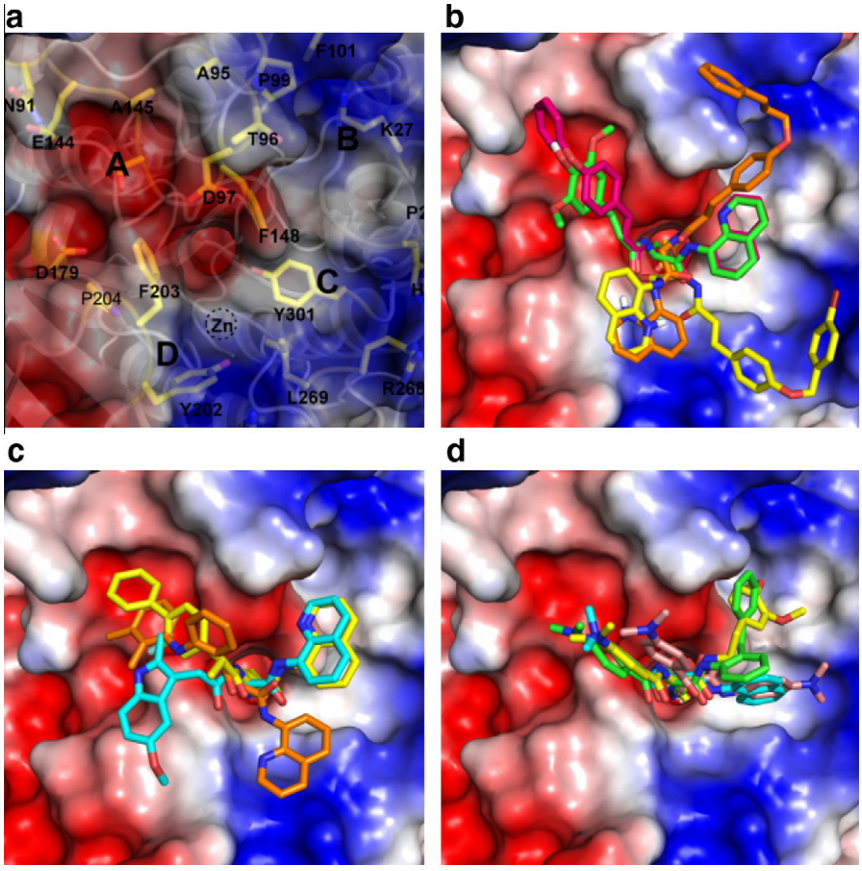


Figure 3. Solvent excluded surface of PfHDAC1 homology model colored by electrostatic potential (blue +, red −); (a) transparent surface showing residues that surround surface pockets A–D; (b) Gold docked cinnamate inhibitors (**13a** green, **13d** magenta, **13h** orange, **13i** yellow); (c) NSAID-derived inhibitors (**13n** yellow, **13p** orange, **13r** lt blue); (d) varied amine-derived inhibitors (**13w** yellow, **13x** pink, **13y** lt blue, **13z** green) shown.

pockets B and C (inhibitors **13w**, **13x**, **13y**, and **13z**, Fig. 3d). The model and docking results suggest that binding of Asu derivatives requires inhibitors to maximize H-bonding to Asp97 and to efficiently fill pockets nearest the active-site entrance. Docking placed side chains of ligands close to residues Ala95 and Thr96 not present in HDAC1, thus posing Thr96 as a polar target for increase selectivity over HDAC1.

Described herein is a series of potent antimalarial compounds, 16 with IC_{50} <100 nM and 5 with IC_{90} <50 nM against a drug sensitive (3D7) strain of *P. falciparum*. Five inhibitors were equipotent or more potent than **Asu-9**, but selectivity was reduced relative to **Asu-13**. Some inhibitors contained NSAIDs as effective HDAC-binding components. The inhibitors showed selectivity in killing the malarial parasite over normal cells and they caused hyperacetylation of *P. falciparum* histones. When docked in a PfHDAC1 homology model, a preferred inhibitor binding mode revealed amide–NH H-bonding to PfHDAC1 Asp97. The inhibitors reported herein share a branch or fork at the chiral center, thereby enabling generation of compound libraries that can potentially distinguish between HDAC surface contours at the entrance to the HDAC active sites, thereby potentially enabling discrimination between different Pf and human HDACs. This may be an important approach to enhancing selectivity over human HDAC enzymes. Together the data supports the case for targeting the PfHDAC1 enzyme to obtain novel antimalarial drugs.

Acknowledgments

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Supplementary data

Supplementary data (experimental methods, compound syntheses, and characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.096.

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