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Identification of novel quinazoline derivatives as potent antiplasmodial agents

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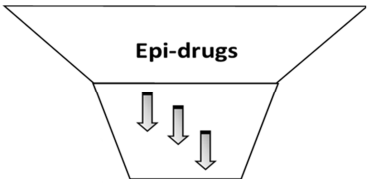
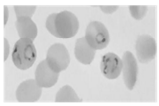
Graphical Abstract

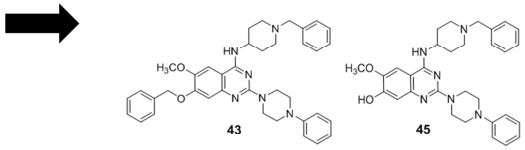


In vitro screening against *P. falciparum*



Toxicity and PK studies

In vivo assay (murine malaria model)
1 HDAC inhibitor and 3 DNMT3a inhibitors selected



Identification of 3 DNMT3a inhibitors as potent antimalarials

Identification of Novel Quinazoline Derivatives as Potent Antiplasmodial Agents

Anne Bouchut [a¶], Dante Rotili [b¶], Christine Pierrot [a], Sergio Valente [b], Sophia Lafitte [a], Johan Schultz [c], Urban Hoglund [d], Roberta Mazzone[b], Alessia Lucidi [b], Giancarlo Fabrizi[b], Dany Pechalrieu[e], Paola B. Arimondo [e], Tina S. Skinner-Adams [f], Ming Jang Chua [f], Kathy T. Andrews [f], Antonello Mai [b,g,*], Jamal Khalife [a,*]

[a]CIIL U1019-CNRS UMR 8204, Université de Lille, Institut Pasteur de Lille, 1 rue du Professeur Calmette, 59019 Lille, France

[b]Department of Drug Chemistry and Technologies, Sapienza University of Rome, 00185 Rome, Italy

[c]Kancera AB, Banvaktsvägen 22, SE-17148, Solna, Sweden

[d]Adlego Biomedical AB, P.O. Box 42, SE-751 03 Uppsala, Sweden

[e]CNRS FRE3600, ETaC, IBCG, 31062 Toulouse cedex 09, France

[f]Tropical Parasitology Lab, Griffith Institute for Drug Discovery, Don Young Road, Griffith University, Nathan, Queensland, Australia 4111

[g]Pasteur Institute, Cenci-Bolognetti Foundation, Sapienza University of Rome, 00185 Rome, Italy.

Corresponding Author

J.K.: phone, +33 320877968; fax, +33 320877888; E-mail, jamal.khalife@pasteur-lille.fr

A.M.: phone, +39 0649913392; fax, +39 0649693268; E-mail, antonello.mai@uniroma1.it

Author Contributions

¶A.B. and D.R. contributed equally to this work.

Abbreviations

DNMT, DNA methyltransferase; DOT1L, disruptor of telomeric silencing 1-like; EZH2, enhancer of zeste homolog 2; HAT, histone acetyltransferase, HMEs, histone modifying enzymes; HMT, histone methyltransferase; HPβCD, (2-hydroxypropyl)-beta-cyclodextrin; Jmj-C, Jumonji-containing demethylases; KDMs, histone demethylases; LSD1, lysine-specific histone demethylase 1A; PARP, poly(ADP-ribose) polymerase; PRMT, protein arginine methyltransferase; WHO, world health organization.

Abstract

Despite the recent reductions in the global burden of malaria, this disease remains a devastating cause of death in tropical and subtropical regions. As there is no broadly effective vaccine for malaria, prevention and treatment still rely on chemotherapy. Unfortunately, emerging resistance to the gold standard artemisinin combination therapies means that new drugs with novel modes of action are urgently needed. In this context, *Plasmodium* histone modifying enzymes have emerged as potential drug targets, prompting us to develop and optimize compounds directed against such epigenetic targets. A panel of 51 compounds designed to target different epigenetic enzymes were screened for activity against *Plasmodium falciparum* parasites. Based on *in vitro* activity against drug susceptible and drug-resistant *P. falciparum* lines, selectivity index criterion and favorable pharmacokinetic properties, four compounds, one HDAC inhibitor (**1**) and three DNMT inhibitors (**37**, **43** and **45**), were selected for preclinical studies in a mouse model of malaria. *In vivo* data showed that **37**, **43** and **45** exhibited oral efficacy in the mouse model of *Plasmodium berghei* infection. These compounds represent promising starting points for the development of novel antimalarial drugs.

Keywords: antimalarial agents; histone deacetylase inhibitors; DNA methyltransferase inhibitors; PK studies; *P. berghei* mouse model.

1. Introduction

Plasmodium falciparum is the most virulent of the malaria parasites that infect humans and is responsible for most of the malaria-related deaths. While the global burden of malaria decreased, according to the latest WHO estimates released, there were still 212 million cases of malaria in 2015 and 429,000 deaths [1]. Although substantial and tangible improvements are being made in the search of an effective vaccine [2-5], antimalarial drugs remain one of the main strategies to prevent and treat malaria. Of concern in this regard is that resistance and/or delayed parasite clearance to most available drugs, including artemisinin-based combination therapies, have appeared [6,7]. Consequently, it is imperative to discover new antimalarial agents that are able to prevent or treat malaria *via* different mechanisms. As most epigenetic enzymes play essential roles in proliferation and differentiation of eukaryotic cells, *Plasmodium* histone modifying enzymes (HMEs) have been proposed as potential drug targets [8-14].

In the *P. falciparum* genome, five histone deacetylase enzymes (HDACs), 10 histone acetylation enzymes (HATs), 13 potential histone methylation enzymes (histone methyltransferases, HMTs, including 10 lysine methyltransferases and three arginine methyltransferases) and three histone demethylase enzymes (KDMs), have been identified (reviewed in [15]). Half of the lysine methyltransferases and one HDAC (P*f*HDAC3/P*f*Hda2) have been shown to be refractory to genetic disruption, suggesting an essential and important role in *Plasmodium* biology [16,17]. Investigation of HMEs as promising drug targets in *Plasmodium* showed that apicidin, curcumin and various hydroxamate derivatives were potent inhibitors of parasite growth *in vitro* [13,16,18-22]. Further work identified the small molecule BIX01294 (**18**), an inhibitor of the H3K9 methyltransferases G9a/GLP [23], as a potent antiplasmodial against blood parasites *in vitro* and *in vivo* [24,25]. The clinically approved HDAC-targeting

cancer drugs vorinostat, romidepsin, belinostat and panobinostat [26] have also been shown to cause hyperacetylation of parasite histones and to inhibit the growth of multiple *P. falciparum* lines *in vitro*. Epigenetic control also includes DNA methylation that has been extensively studied as a stable epigenetic marker [27]. In mammalian cells, the role of C5 DNA methyltransferases (DNMTs) is essential for cell development and survival [28-33]. Hence DNMTs have been investigated as cancer drug targets and two inhibitors, azacitidine and decitabine, have been approved by the Food and Drug Administration and the European Medicines Agency for use against myelodysplastic syndrome, acute myeloid leukemia and chronic myelomonocytic leukemia [34,35]. In *P. falciparum*, a functional DNMT (PF3D7_0727300) has been partially characterized, with peak of expression in early/late trophozoites (PlasmoDB [36]). More recent genome-wide mapping showed the methylation of *P. falciparum* DNA, the hypomethylation of core promoters and a sharp methylation at exon-intron boundaries and nucleosomes [37].

The above observations underscore the importance of histone and DNA modifying enzymes to *Plasmodium* parasite survival and support the concept that these enzymes could represent drug targets worthy of further exploration. In this study, compounds initially designed to target different epigenetic enzyme families in eukaryotes, such as histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone demethylases (KDMs), DNA methyltransferases (DNMTs) and poly(ADP-ribose) polymerases (PARPs), were assessed for growth inhibitory activity against *Plasmodium* 3D7 parasites *in vitro*. After a first screening of epi-drugs, a focused screening has been performed with analogues of the first hit compounds. The best-scoring compounds from the two screenings were then assayed against the multi-drug resistant *P. falciparum* lines W2 and Dd2 to determine their potency, and against mammalian cells to assess their cytotoxicity and selectivity indexes. From these results,

four compounds were selected to perform pharmacokinetic (PK) analysis and *in vivo* studies on *P. berghei*-infected mice.

2. Results

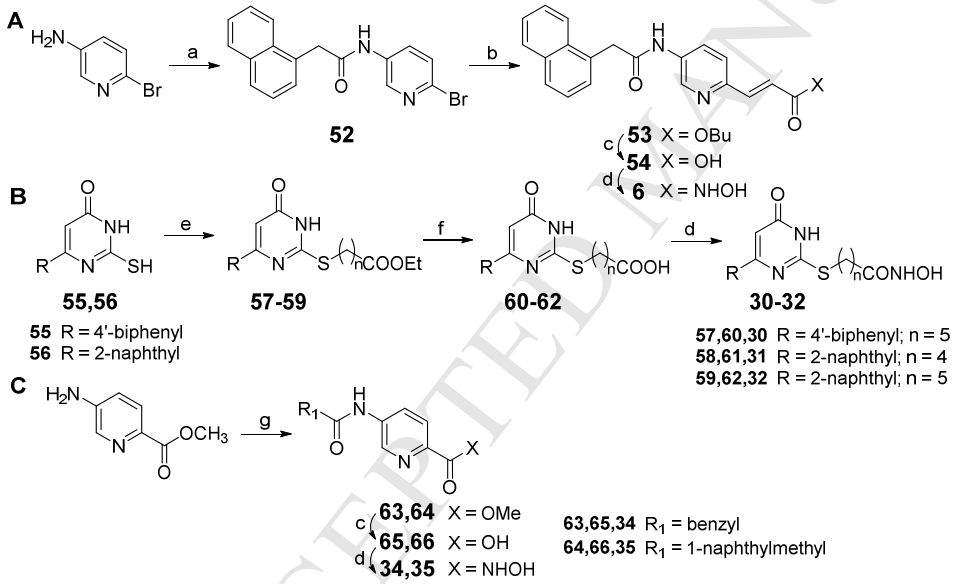
2.1. Chemistry

Among the epi-drugs used for the first screening (Table 1) only **6**, a *N*-hydroxy-3-pyridin-2-ylacrylamide designed as a HDAC inhibitor, was not already described. For the synthesis of **6**, 2-bromopyridin-5-amine was converted into the corresponding amide intermediate **52** through reaction with 1-naphthylacetyl chloride in dry dichloromethane in the presence of triethylamine. Afterwards, **52** was dissolved in dry *N*,*N*-dimethylformamide and treated with tetra-*n*-butylammonium iodide, sodium acetate trihydrate, butyl acrylate and, under nitrogen atmosphere, triphenylphosphine and palladium acetate (Scheme 1A). The resulting mixture was heated in a sealed tube at 140 °C providing the butyl acrylate **53**, which was converted into the corresponding carboxylic acid **54** through basic hydrolysis (lithium hydroxide in tetrahydrofuran/water) and finally into the hydroxamate **6** by i) activation with ethyl chloroformate and triethylamine in dry tetrahydrofuran at 0 °C, ii) nucleophilic displacement with *O*-(2-methoxy-2-propyl)hydroxylamine in dry tetrahydrofuran at 0 °C, and iii) cleavage with Amberlyst 15 ion-exchange resin in methanol (Scheme 1A).

Three analogues of **1** (**30**-**32**) and two analogues of **6** (**34**, **35**) were synthesized and tested in the focused screening (Table 2), to improve their antimalarial activity. The synthetic route followed for the preparation of compounds **30**-**32** is depicted in Scheme 1B. The known 2-mercapto-6-substituted-pyrimidin-4(3*H*)-ones **55** [38] and **56** [39] were treated with the appropriate commercially available ethyl ω-bromoalkanoate in presence of anhydrous potassium

carbonate to afford the ethyl esters **57**-**59**, which were then hydrolyzed to the related carboxylic acids **60**-**62** by the means of potassium hydroxide in ethanol at room temperature (rt), and then converted into the hydroxamates **30**-**32** (Scheme 1B) following the procedure used for the synthesis of **6**.

The syntheses of **34** and **35** were accomplished starting from methyl 5-aminopicolinate acylated with the appropriate acyl chlorides to give the intermediate amides **63** and **64**. After hydrolysis of **63** and **64** under basic condition to the corresponding carboxylic acids **65** and **66**, the compounds were converted into the hydroxamates **34** and **35** by the usual way (Scheme 1C).

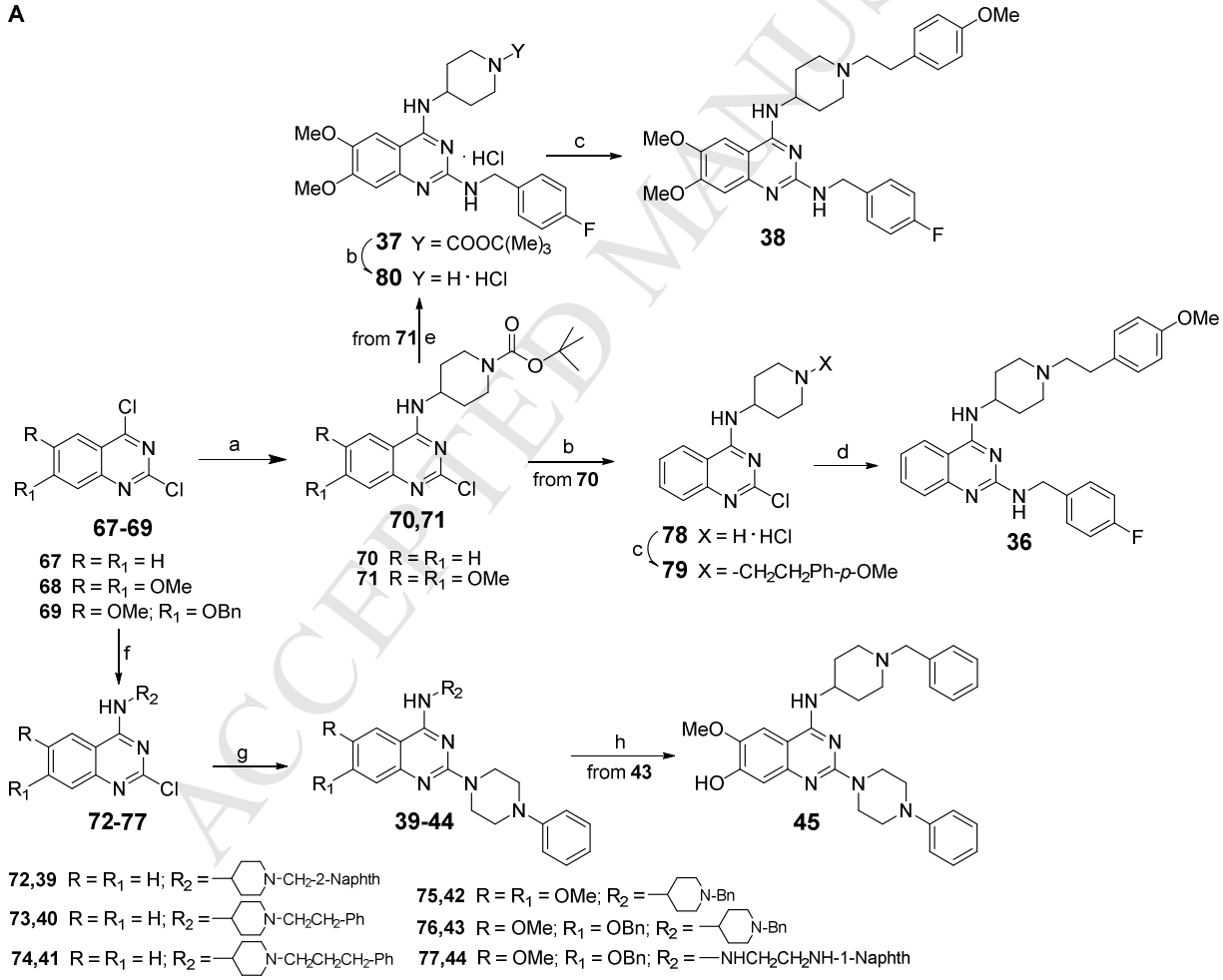


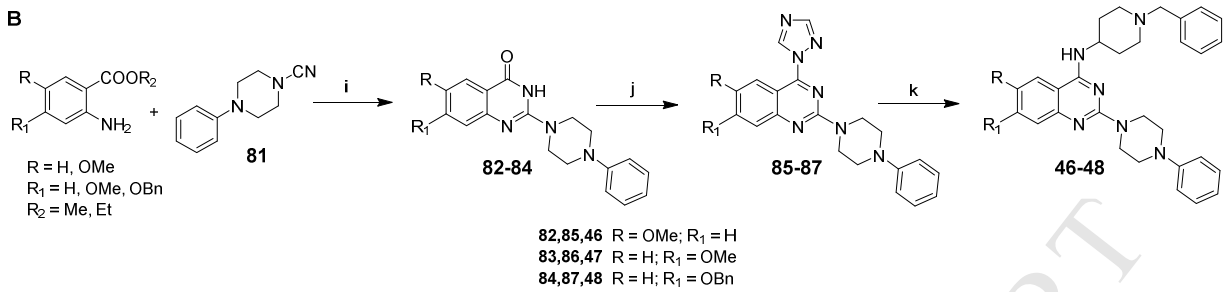
Scheme 1. Synthesis of the HDACi **6**, **30**-**32**, **34**, **35**. Reagents and conditions: (a) 1-naphthylacetyl chloride, dry DCM, triethylamine, rt, 2 h; (b) triphenylphosphine, palladium acetate, tetra-*n*-butylammonium iodide, sodium acetate trihydrate, butyl acrylate, dry DMF, 140 °C, sealed tube, overnight; (c) lithium hydroxide, ethanol, rt, overnight; (d) 1) ethyl chloroformate, triethylamine, dry THF, 0 °C to rt, 10 min; 2) $NH_2OC(CH_3)_2OCH_3$, dry THF, 0 °C to rt, 15 min to 3 h; 3) Amberlist 15, methanol, rt, 1-2 h; (e) appropriate ethyl ω-

bromoalkanoate, anhydrous potassium carbonate, dry DMF, rt, 1 h; (f) 2N potassium hydroxide, ethanol, rt, 18 h; (g) appropriate acyl chloride, dry DCM, triethylamine, rt, 2 h.

Since the quinazoline **28** [40] was identified in the first screen as a hit compound against *P. falciparum* 3D7, some analogues (**36**-**48**) were synthesized and tested as potential DNMT inhibitors and antimalarials (see Table 4 for structures). Scheme 2 shows the general synthetic pathway followed for the preparation of the final derivatives **36-45**. The known 2,4-dichloroquinazolines **67** [41], **68** and **69** [42] were subjected to a displacement reaction at the C4 position with the appropriate commercial amines, generating the 2-chloro-4-aminosubstituted quinazoline intermediates **70** and **71** [43], **72-74**, **75** [44], **76** and **77** [45]. The 2-chloroquinazoline **70**, dissolved in a mixture of dry THF and dry methanol, was treated with 4N hydrochloric acid in dioxane providing the 2-chloro-*N*-(piperidin-4-yl)quinazolin-4-amine hydrochloride **78**, which underwent an alkylation reaction with commercial 4-methoxyphenethyl bromide in the presence of potassium carbonate and sodium iodide in dry DMF at rt to give the intermediate **79**. Further C2-chloro displacement on **79** with 4-fluorobenzylamine in a sealed tube at 110 °C afforded the final compound **36** (Scheme 2A). C2-Chloro displacement performed on the 2-chloro-6,7-dimethoxyquinazoline **71** with 4-fluorobenzylamine in *iso*-propyl alcohol at 130 °C under microwave irradiation afforded **37**. Compound **37** was then converted into **80** via BOC-deprotection performed with 4N hydrochloric acid in dioxane and transformed into **38** by alkylation with 4-methoxyphenethyl bromide in the presence of potassium carbonate and sodium iodide in dry DMF at rt (Scheme 2A). The final compounds **39**-**44** were obtained by reacting the 4-amino-2-chloroquinazolines **72**-**77** with *N*-phenylpiperazine in a sealed tube at 110 °C (Scheme 2A). The 7-hydroxyquinazoline **45** was prepared through debenzylation reaction of **43** with trifluoroacetic acid under reflux conditions (Scheme 2A).

Final compounds **46-48** were prepared as reported in Scheme 2B. The 2-phenylpiperazine-6,7-substituted-quinazolinones **82**, **83** and **84** [46] were synthesized via cyclization reaction between the appropriate commercial ethyl/methyl anthranilates and the *N*-phenylpiperazine-1-carbonitrile **81** [45,46], in the presence of dry sodium hydride in xylene at 140 °C under nitrogen atmosphere. The reaction between **82**-**84** and 1,2,4-triazole in the presence of phosphorous oxychloride and triethylamine produced the triazolyl-quinazolines **8**5-**87** [46] which reacted with 4-amino-1-benzylpiperidine in dry dioxane at 110 °C to provide the final compounds **46-48**.





Scheme 2. Synthesis of the new quinazoline derivatives **36-48**. Reagents and conditions: (a) *tert*-butyl 4-aminopiperidine-1-carboxylate, triethylamine, dry THF, 0 °C to rt, 4-72 h; (b) 4N hydrochloric acid in dioxane, dry THF/dry methanol 1:1, 0 °C to rt, 7-25 h; (c) 4-methoxyphenetyl bromide, dry potassium carbonate, sodium iodide, dry DMF, rt, 19-27 h; (d) 4-fluorobenzylamine, *iso*-amyl alcohol, 110 °C, sealed tube, 8 h; (e) 4-fluorobenzylamine, *iso*-propyl alcohol, microwave, 130 °C, 5 h; (f) appropriate amine, triethylamine, dry THF, 0 °C to rt, 4-6.5 h; (g) *N*-phenylpiperazine, *iso*-amyl alcohol, 110 °C, sealed tube, 4 h; (h) trifluoroacetic acid, 0 °C to 115 °C, 35 min; (i) dry sodium hydride, xylene, N_2, 140° C, 3-4 h; (j) (1) phosphorus oxychloride, triethylamine, 1,2,4-triazole, dry acetonitrile, 0° C (40 min) then rt (30 min); (2) addition of a solution of quinazolinone in dry acetonitrile (or dry chloroform), N_2, rt, overnight then reflux, 2-5 h; (k) 4-amino-1-benzylpiperidine, *N*,*N*-di*iso*-propylethylamine, dry dioxane,110° C, 20-24 h.

General procedures for the syntheses of the HDACi **6**, **30**-**32**, **34**, **35** and of the DNMT3a inhibitors **36**-**48**, as well as their chemical, physical and spectral (^{1}H- and ^{13}C-NMR) data are reported in Supplementary material. Chemical, physical and spectral (^{1}H-NMR) data of the intermediate unknown (*i.e.* previously not characterized) compounds **52-54**, **57-66**, **72-74**, **78-80**,

82, **83**, **85**, and **86** are reported in Table S1 in Supplementary material. Elemental analyses for compounds **6**, **30**-**32**, **34**, **35**, **36-48** are reported in Table S2 in Supplementary material.

2.2. Biochemistry

The *N*-hydroxy-3-(5-(2-(naphthalen-1-yl)acetamido)pyridin-2-yl)acrylamide **6** as well as the analogues of **1**, **30**-**32,** and the analogues of **6, 34** and **35,** were tested against human HDAC1, HDAC4 and HDAC6 (representative of class I, class IIa, and class IIb HDACs, respectively) to determine their potency and selectivity. Trichostatin A (TSA) and TMP269 [47] were used as reference drugs.

Data in Table S3 (Supplementary material) showed that all the new synthesized HDACi displayed submicromolar inhibition against HDAC1 and nanomolar inhibition against HDAC6, while they were much less potent (if at all) against HDAC4.

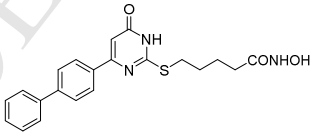
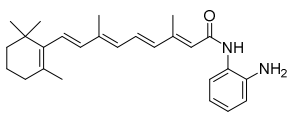
The new quinazoline derivatives **36-48**, analogues of the DNMT3a-selective **28** [40], were tested against active human recombinant DNMT1, DNMT3a and G9a to determine their half maximal effective concentrations (EC_{50}). Data presented in Table S4 (Supplementary material) showed that the tested compounds **36-43**, **45-47** were more potent (EC_{50} between 1.6 and 8.7 µM) against DNMT3a than against the other tested methyltransferases, with the different chemical substitutions at the C2, C4, C6, and C7 quinazoline positions having only modulatory effects on the potency of derivatives. Compounds **44** and **48** were less potent against DNMT3a (% inhibition at 10 µM <50%), thus they were not tested against DNMT1 and G9a.

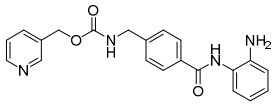
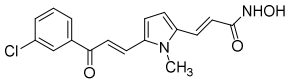
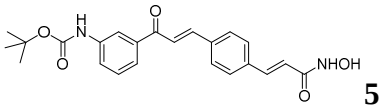
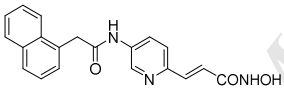
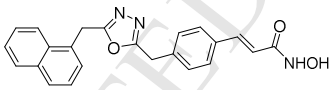
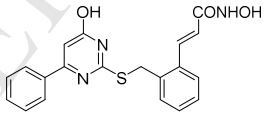
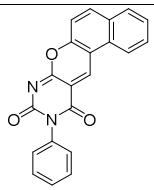
2.3. Phenotypic screening of epigenetic modulators against asexual intraerythrocytic stage of P. falciparum *3D7 parasites*

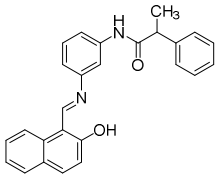
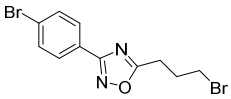
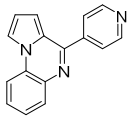
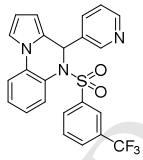
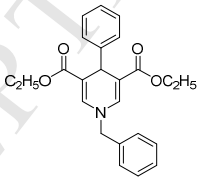
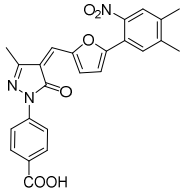
The antiplasmodial activities of the first 29 compounds targeting different classes of epigenetic targets were screened for activity against drug-sensitive *P. falciparum* 3D7 parasites at the single concentration of 10 µM. As shown in Table 1, 22 compounds inhibited parasite growth by at least 50% and seven compounds inhibited parasite growth by less than 25%. The 22 compounds giving >50% inhibition were then tested in dose response assays to determine their IC_{50} values (50% or half maximal inhibitory concentration). The most potent compounds (IC_{50} <50 nM) were two hydroxamate-based HDAC inhibitors, **1** (IC_{50} = 4.0 nM) and **6** (IC_{50} = 10.2 nM), both with selectivity for class I/IIb human HDACs, and the G9a/GPL inhibitor **18** (IC_{50} = 20.8 nM), for which similar activity has been previously reported [24]. Submicromolar inhibition of *Plasmodium* growth was also displayed by **19** [48] (UNC0638, IC_{50} = 67.9 nM), a G9a/GLP inhibitor related to **18**, and by the recently reported DNMT3a inhibitor **28** [40] (IC_{50} = 325.5 nM) (Table 1).

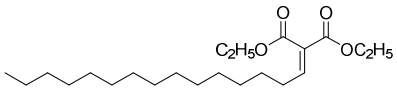
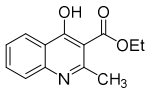
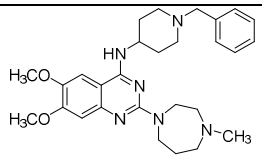
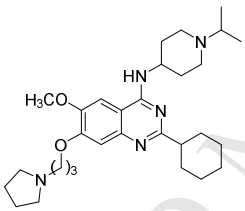
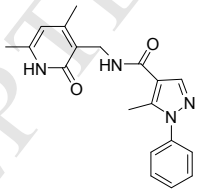
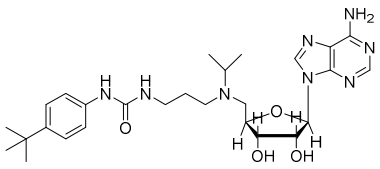
Table 1

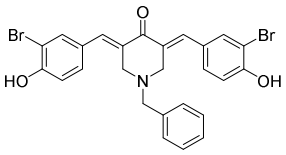
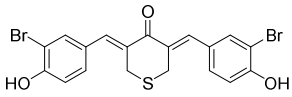
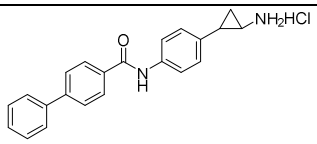
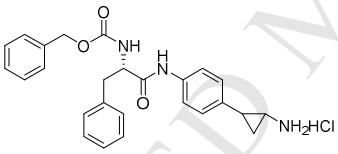
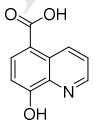
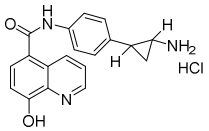
In vitro antiplasmodial activity of compounds against *P. falciparum* 3D7-infected erythrocytes.

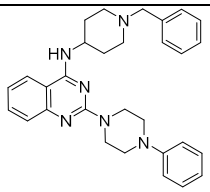
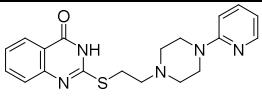
Epi-target[a]	**Compound**	**% inhibition at 10 µM**	**IC_{50} (nM)**[b]
HDAC	 **1** [49] class I/IIb HDACi	97.0	4.0 ± 0.3
	 **2** [50] context-selective class I	97.1	>1 µM

HDACi			
 **3** [51]	HDAC1-3 inhibitor	50.9	>1 µM
 **4** [52]	HDAC4-6,8 inhibitor	89.6	>1 µM
 **5** [53]	HDAC6-selective inhibitor	100	422.4 ± 19.4
 **6**	class I/IIb HDACi	96.8	10.2 ± 2.6
 **7** [54]	pan-HDACi	98.0	244.0 ± 52.2
 **8** [55]	HDAC8 inhibitor	2.8	ND[c]
Sirtuin	 **9** [56, 57] Sirt1/2i	80.5	>1 µM

	 **10** [58, 59] Sirt1/2i	72.3	>1 µM
	 **11** [60] Sirt2-selective inhibitor	18.7	ND
	 **12** [61] Sirt6 activator	70.7	>1 µM
	 **13** [61] Sirt6 activator	99.5	671.2 ± 15.5
	 **14** [62, 63] Sirt1 activator	97.2	>1 µM
HAT	 **15** [64, 65] p300 inhibitor	12.8	ND

	 **16** [66, 67] p300 inhibitor/PCAF activator	92.7	>1 µM
	 **17** [68] p300/CBP inhibitor	60.1	>1 µM
HMT	 **18** [23-25] G9a/GLP inhibitor	100	20.8 ± 4.9
	 **19** [48] G9a/GLP inhibitor	100	67.9 ± 0.1
	 **20** [69] EZH2 inhibitor	14.7	ND
	 **21** [70] DOT1L inhibitor	9.7	ND

	 **22** [71, 72] PRMT4/EZH2/HAT inhibitor (multi-target)	100	> 1µM
	 **23** [71, 72] PRMT1 inhibitor	99.5	951.9 ± 86.1
KDM	 **24** [73] LSD1 inhibitor	97.5	>1 µM
	 **25** [73] LSD1 inhibitor	64.6	>1 µM
	 **26** [74] Jmj-C inhibitor	25.4	ND
	 **27** [75] pan (LSD1+Jmj-C) KDM inhibitor	100	>1 µM

DNMT	 **28** [40] DNMT3a inhibitor	100	325.5 ± 22.1
PARP	 **29** [76] PARP1 inhibitor	2	ND

[a]Epi-target, epigenetic modulator target as identified for human cells/targets. [b]IC_{50} values were not determined when the percentage of inhibition at 10 µM was lower than 50%. [c]ND, Not Determined.

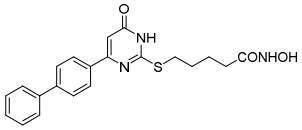
2.4. Antiplasmodial activity of analogues derived from active compounds

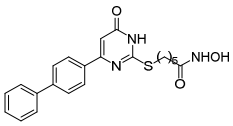
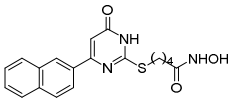
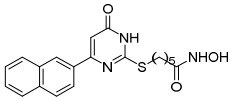
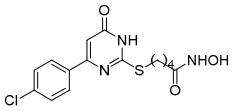
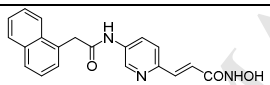
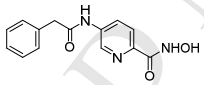
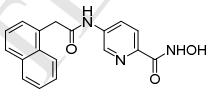
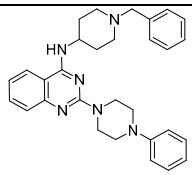
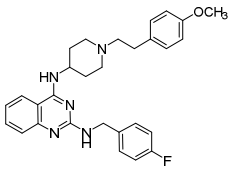
In order to optimize the potency of active compounds, 22 further analogues of previous hit compounds were tested. The four analogues of **1** had different C6-uracil substitutions [2-naphthyl (**31**, **32**) or 3-chlorophenyl (**33**) instead of the 4-biphenyl group], and/or different lengths of spacers between the sulphur atom and the hydroxamide [five (**30, 32**) instead of four methylene groups]. The two analogues of **6** had their *N*-hydroxyacrylamide group replaced with a *N*-hydroxycarboxamide group. Compound **34** also had its 1-naphthyl moiety substituted with a phenyl ring. All **1** and **6** analogues showed selectivity for human class I/IIb HDACs analogously to their prototypes (Table S3). In addition, 13 DNMT inhibitors analogues of **28** were prepared and tested, with changes at quinazoline C2 (*p*-fluorobenzylamine or phenylpiperazine), C4 (*N*-arylalkylsubstituted 4-aminopiperazine, *N-tert*-butoxycarbonyl 4-aminopiperazine, 2-(1-naphthylamino)ethylamine), C6 (H, methoxy group), and/or C7 (H, methoxy, benzyloxy group)

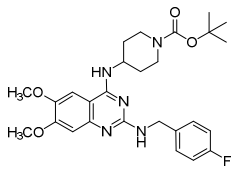
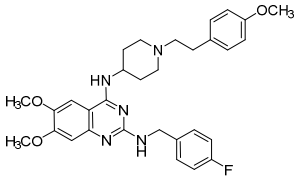
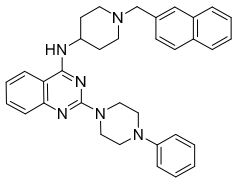
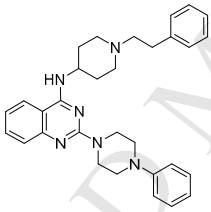
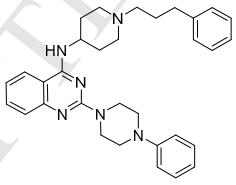
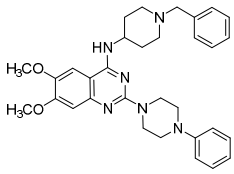
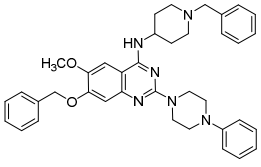
position. Finally, as **27** gave 100% inhibition at 10 µM in the primary screen (Table 1), three pan-KDM inhibitors (analogues of **27**) were also included. These 22 analogues were first screened at 10 µM against *P. falciparum* 3D7 and, with the exception of **49**, all displayed 96-100% inhibition of parasite growth. Compound **49**, a pan-KDM inhibitor, was excluded from further analysis. For the remaining 21 analogues, IC_{50} values were then determined as previously described (Table 2). Among the HDACi examined, the analogues of **1** (compounds **30**-**33**) exhibited IC_{50} values <100 nM while the analogues of **6** (compounds **34**, **35**) were much less potent (IC_{50} >1 µM). Nevertheless, none of the compound among the two groups showed IC_{50} values lower than the corresponding prototypes (Table 2). Among the tested DNMT inhibitors, all 13 analogues of **28** showed lower IC_{50} values than the prototype, with **36**-**38**, **43** and **45** having a ~10-32-fold lower IC_{50} than **28** (Table 2, Fig. 1). In the case of the analogues of the pan-KDM inhibitor **27**, all tested analogues displayed increased IC_{50} values (>1µM). Based on data of both screenings, we selected the two HDACi **1** and **6**, the G9a inhibitors **18** and **19**, and the DNMT inhibitors **37, 38, 43, 45** for further investigation.

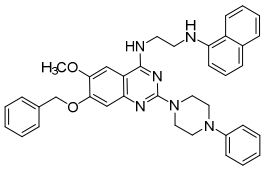
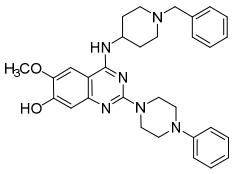
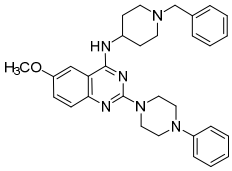
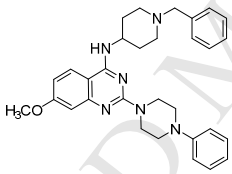
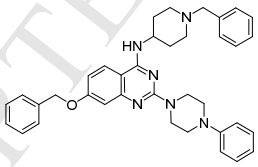
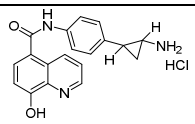
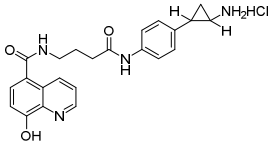
Table 2

In vitro antiplasmodial activity of hits (*Pf*3D7 IC_{50} <1 µM) and analogues against *P. falciparum* 3D7-infected erythrocytes.

Compound		**% inhibition at 10 µM**	**IC_{50} (nM)**[a]
HDACi (analogues of **1**) class I/IIb HDACi	 **1** [49]	96.1	6.0 ± 2.1

	 **30**	97.4	50. 9 ± 5.0
	 **31**	97.9	25.2 ± 5.0
	 **32**	97.4	79.4 ± 0.7
	 **33**	98.0	88.6 ± 2.2
HDACi (analogues of **6**) class I/IIb HDACi	 **6**	97.9	21.1 ± 2.4
	 **34**	97.8	>1 µM
	 **35**	97.9	>1 µM
DNMT3a inhibitors (analogues of **28**)	 **28** [40]	100	325.5 ± 22.1
	 **36**	100	33.6 ± 8.7

	100	
37		18.7 ± 0.9
	100	
38		18.0 ± 5.4
	100	138.2 ± 22.1
39		
	100	98.9 ± 13.7
40		
	100	75.1 ± 6.2
41		
	100	37.8 ± 6.3
42		
	100	34.0 ± 19.0

43 		99.6	56.2 ± 0.3
44 		100	10.1 ± 5.1
45 		100	52.5 ± 6.7
46 		100	83.0 ± 16.3
47 		100	66.9 ± 6.9
48			
pan-KDM inhibitors (analogues of **27**)	 **27** [75]	98.9	775.8 ± 293
	 **49** [75]	45.3	ND[b]

50 [75]		97.8	>1 µM
51 [75]		96.7	>1 µM

[a]IC_{50} values were not determined when the percentage of inhibition at 10 µM was lower than 50%. [b]ND, not determined.

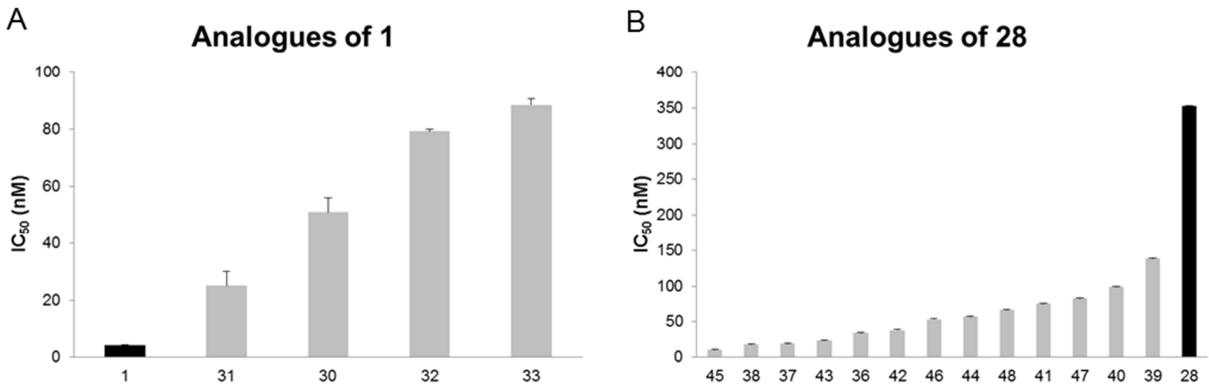


Fig. 1. Hit compounds and analogues selected for further investigation. Each histogram shows one candidate and its analogues (X axis). The parent hit compound of each series is shown in black, the analogues are in grey. Results are shown as mean ± SD for 2 independent assays.

2.5. Antiplasmodial activity against multi-drug resistant P. falciparum *lines W2 and Dd2*

Next, compounds were assessed for growth inhibitory activity against the multidrug-resistant *P. falciparum* lines W2 and Dd2 (resistant to chloroquine, quinine, pyrimethamine and sulfadoxine) [78,79] and the activities were compared to those obtained against the drug-sensitive

3D7 line. Under the experimental conditions of the present study, chloroquine had IC_{50} values of 21.5 nM (± 1.6) and 290.0 nM (± 6) for 3D7 and W2 respectively, consistent with previous reports [80-82]. Of the eight compounds spanning HDAC, G9a, and DNMT inhibitor classes, activity against W2 was found to be similar to that against 3D7, with IC_{50} values <50 nM for all tested compounds (Table 3). Interestingly, **19** was more potent against the multidrug-resistant W2 than against the wild-type 3D7 line. Similar IC_{50} values to 3D7 were obtained for all compounds when tested against Dd2 using the [^{3}H]-hypoxanthine uptake assay method (Table 3). Together, these data show that these eight compounds have early lead (IC_{50} <100 nM) profiles [83] with respect to their potency, and demonstrate a lack of cross-resistance to multi-drug resistant *P. falciparum* lines.

Table 3

Comparative *in vitro* activity (IC_{50}) of selected compounds against drug-sensitive *P. falciparum* line 3D7 versus multidrug-resistant *P. falciparum* lines Dd2 and W2.

Epi-target[a]	**Compound**	***P. falciparum* IC_{50} (nM)**		
		3D7 (n=3)	**Dd2** (n=3)	**W2** (n=2)
HDAC	**1**	4.2 ± 0.4	4.0 ± 1.1	6.6 ± 0.1
HDAC	**6**	13.3 ± 5.6	13.5 ± 6.9	15.3 ± 0.8
G9a	**18**	23.7 ± 6.1	9.7 ± 1.6	16.7 ± 8.7
G9a	**19**	68.7 ± 1.4	16.8 ± 6.3	38.5 ± 4.0
DNMT3a	**37**	18.9 ± 0.9	18.5 ± 3.8	43.6 ± 10.4
DNMT3a	**38**	18.0 ± 5.4	6.1 ± 3.7	25.8 ± 5.3
DNMT3a	**43**	34.0 ± 18.6	81.3 ± 44.3	39.9 ± 9.9

DNMT3a	**45**	10.1 ± 5.1	42.6 ± 12.0	18.6 ± 8.4

[a]Epi-target, epigenetic modulator target as identified for human cells/targets.

2.6 Plasmodium *specific selectivity versus primary activated cells (splenic murine cells) and a eukaryotic cell line (HFF)*

To determine the selectivity Index (SI) of the eight selected compounds (Table 3), cytotoxicity was assessed on murine splenic primary cells as well as human foreskin fibroblasts (HFF) and IC_{50} values were compared to those obtained for *P. falciparum* growth inhibition. As shown in Table 4, all compounds showed SI >100 against the parasites versus HFFs, except for **19** which displayed the lowest selectivity (SI = 33). The SI against primary murine cells was between 44 and >500, with the exception of the HDACi **6** (SI = 9) and the G9a inhibitors **18** and **19** (SI = 23 and 21, respectively).

Table 4

Compound selectivity for parasite versus mammalian cells.

Epi-target[a]	**Compound**	***Pf*3D7**	**Mammalian cells**			
			Splenic cells		**HFF**	
		IC_{50} (nM)	**IC_{50} (nM)**	**SI**[b]	**IC_{50} (nM)**	**SI**
HDAC	**1**	4.2 ± 0.4	785 ± 78	186	3,295 ± 247	783
HDAC	**6**	13.3 ± 5.6	120 ± 42	9	>10,000[c]	>750
G9a	**18**	23.7 ± 6.172	555 ± 177	23	4,997 ± 355	211
G9a	**19**	68.7 ± 1.4	1,440 ± 141	21	2,286 ± 659	33

DNMT3a	**37**	18.9 ± 0.9	>10,000	>500	7,170 ± 84	377
DNMT3a	**38**	18.0 ± 5.4	800 ± 141	44	3,215 ± 389	178
DNMT3a	**43**	34.0 ± 18.6	>10,000	>295	5,629 ± 27	166
DNMT3a	**45**	10.1 ± 5.1	2,585 ± 346	255	7,210 ± 14	712

[a]Epi-target, epigenetic modulator target as identified for human cells/targets. [b]SI, selectivity index: mammalian cell IC_{50}/*P. falciparum* IC_{50}. [c]IC_{50} values could not be precisely determined (>10,000 nM) when the highest concentration used was not toxic for mammalian cells.

2.7. Pharmacokinetic analysis

Four compounds were further selected for PK studies: the HDAC inhibitor **1** and the DNMT inhibitors **37**, **43** and **45**. A (2-hydroxypropyl)-β-cyclodextrin (HPβCD)-based formulation, suitable for both intravenous (iv) and *per os* (*p.o.*; oral) administration, was used for PK studies. For each compound, two mice were administered with a single dose (50 mg/kg for **1**, **43** and **45**, and 10 mg/kg for **37**), either iv or *p.o.* by gavage. Plasma samples were analysed and concentrations are summarized in Table 5. Compound **1** administered at a 50 mg/kg dose was well tolerated. The 15 min iv samples showed high (>10 µM) **1** plasma concentration, but decreasing rapidly with time. The *p.o.* and iv plasma samples at 1 h post administration showed that the concentration of **1** was between 0.5 and 2.2 µM and after 3 h between 43 and 300 nM. The 50 mg/kg dose of **43** was well tolerated when administered *p.o*. The same dose administered iv was fatal for the mice. The two mice administered with **43** *p.o.* showed plasma concentrations below 1 µM at all-time points. The 50 mg/kg dose of **45** was well tolerated when administered *p.o*. However, the same dose administered iv led to severe acute toxicity and death so that plasma samples could not be collected. The two mice administered with **45** *p.o.* showed plasma

concentrations of 1-5 µM at 30 and 60 min after administration. After 180 min, the plasma concentration was 0.28-0.5 µM. The poor solubility of **37** only allowed a relatively low concentration formulation (1 mg/mL) to be prepared. The 10 mg/kg dose of **37** was well tolerated when administered *p.o.* The two mice treated with **37** iv showed plasma concentrations of 1-5 µM at the 15 and 60 min, and ~ 0.5 µM after 180 min. The two mice treated with **37** *p.o.* showed very low plasma concentrations at all-time points.

Table 5

Pharmacokinetic parameters of the four selected compounds administered intravenously or orally to mice

Compound/Dose	**Administration route**					
	iv			***per os***		
	Time (min)	**Concentration (µM)**		**Time (min)**	**Concentration (µM)**	
		Mouse 1	**Mouse 2**		**Mouse 3**	**Mouse 4**
1 50 mg/kg	15	15.2	10.7	30	2.43	0.67
	60	2.16	1.67	60	0.90	0.48
	180	0.044	0.043	180	0.30	0.070
37 10 mg/kg	15	3.41	5.11	30	0.13	0.12
	60	1.11	0.93	60	0.28	0.10
	180	0.22	0.46	180	0.03	0.03
43 50 mg/kg	15	Toxic		30	0.38	0.94
	60			60	0.48	0.70
	180			180	0.39	0.28

45 50 mg/kg	15	Toxic	30	4.57	3.43
	60		60	3.52	1.04
	180		180	0.67	0.29

2.8. In vivo antimalarial activity in P. berghei *infected mice*

For the *in vivo* experiments, the doses of the compounds were defined on the basis of their high potency, low toxicity and solubility for oral administration that provide plasma concentrations comparable to the IC_{50s} obtained *in vitro*. The oral efficacy of the four selected compounds was examined using the Peters 4-day suppressive test [84]. Groups of six BALB/c mice were infected intraperitoneally (ip) with 10^6 *P. berghei* ANKA parasites and treated as depicted in Fig. 2A. On day 4 post infection (pi), the peripheral blood parasitemia of all mice in the treated groups was compared to mice in vehicle control groups. None of the mice administered with **1** *p.o.* at 50 mg/kg, showed a significant reduction in parasitemia as compared to control mice (Fig. 2B, Exp. 1, p=0.229). As PK studies revealed that concentrations of **1** are higher in the plasma after 15 and 60 min when administered iv (≥10.7 and ≥1.7 µM, respectively) compared to *p.o.* (≥0.7 and ≥0.5 µM, respectively, Table 5) the efficacy of **1** was also assessed using a combination of oral and iv administration. However, this combined administration route (twice a day, one *p.o.* and one iv), was not effective on blood stage infection (Fig. 2B; Exp. 2).

Oral efficacy of **37** was evaluated at 20 mg/kg because quality control studies revealed that **37** presented some chemical instability and poor solubility at higher concentrations. At day 4 pi, parasites were undetectable in the **37**-treated mice in two independent experiments whereas the vehicle control group showed a parasitemia of ~6% and ~4% in experiment 1 and 2, respectively (Fig. 2C). However, parasites were detected in treated animals from day 6-7 pi (Supplementary

material, Fig. S1A, Exp 1), suggesting that while the 4-day treatment was able to delay the onset of patent parasitemia, it was not sufficient to cure mice. This is supported by data showing that an additional treatment at day 5 pi extended the suppressive effect (Fig. S1A, Exp 2).

When **43** was administered at 50 mg/kg *p.o.* twice a day for four days, it was too toxic (four mice died, the two remaining mice showed no parasites at day 4 pi but one more mouse died at day 10 pi (data not shown)). When **43** was tested at 25 mg/kg *p.o.* twice a day for four days, no adverse symptoms were observed and peripheral blood parasitemia was not detectable in the **43**-treated mice in two independent experiments compared to the vehicle-treated mice (parasitemia of ~6% and ~4% on day 4 pi in experiment 1 and 2, respectively; Fig. 2D). Moreover, parasitemia remained sub-patent until 14 days pi in experiment 1 and was ~2% in experiment 2 (Fig. S1C). When **43** was evaluated at 10 mg/kg *p.o.*, mice treated twice a day for four days had detectable parasites on day 4 pi, however parasitemia was significantly lower ($p<0.002$) compared to the control group (Fig. S1D).

With respect to **45**, its oral administration at 50 mg/kg twice a day for four days reduced blood parasites at day 4 pi in two independent experiments (Fig. 2E). Indeed, infections were sub-patent in mice treated with **45** until day 8 pi and parasitemia remained lower than 10% until the end of each experiment (vehicle control mice reached a parasitemia of ~58% and 31% in experiment 1 and 2, respectively; Fig. S1B).

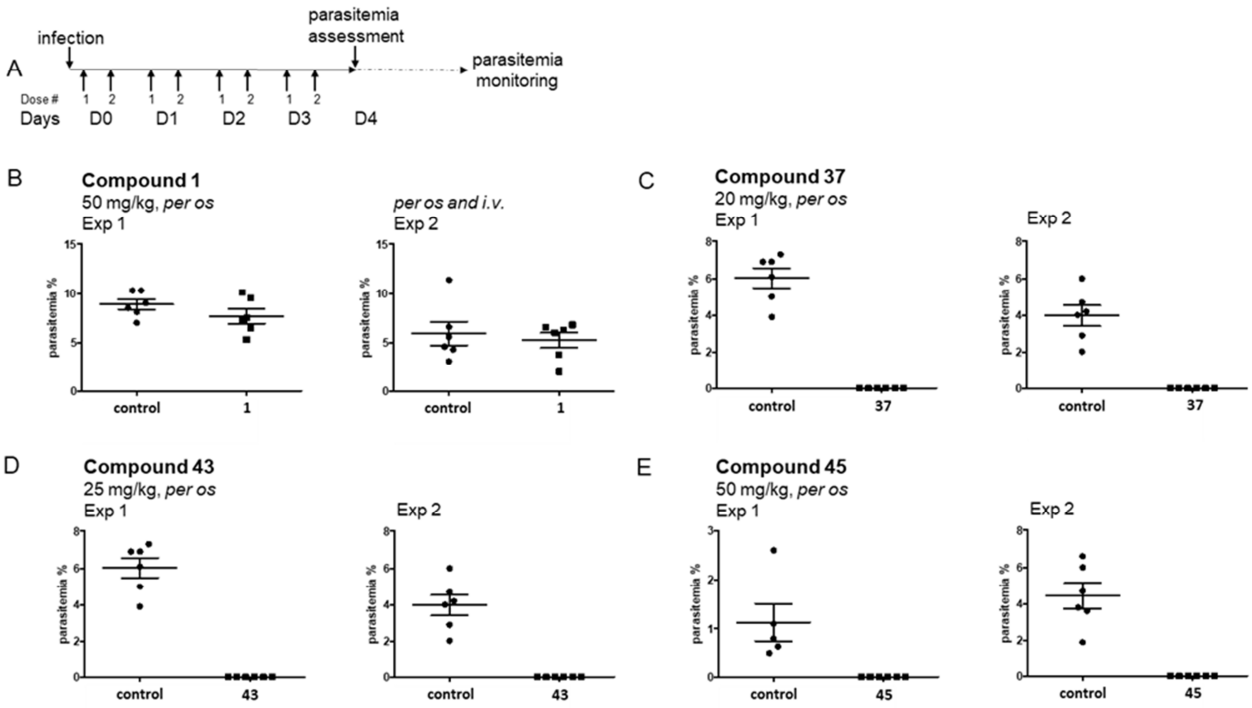


Fig. 2. *In vivo* efficacy of **1**, **37**, **43** and **45** in a murine malaria model. (A) Schematic overview of the experimental protocol (see Experimental in Supplementary material). Parasitemia at day 4 pi for the control-treated groups and for mice treated with compound: (B) **1** (50 mg/kg); (C) **37** (20 mg/kg); (D) **43** (25 mg/kg); (E) **45** (50 mg/kg). The data in panels B, C, D and E are means ± SEM. Each data point represents one mouse.

3. Discussion and conclusion

In an effort to improve the development of *Plasmodium* specific inhibitors of epigenetic processes, we investigated the anti-*Plasmodium* activity of 51 potential inhibitors of histone and DNA modifying enzymes. Our data demonstrate that eight of these compounds exhibit potent activity against drug-sensitive and multidrug resistant *P. falciparum* parasites and have low toxicity towards mammalian control cells. Importantly, these compounds are anticipated to have activity against a range of different eukaryotic epigenetic enzymes.

Among HDAC inhibitors, **1** and **6**, the only two tested HDACi selective towards classes I and IIb HDACs, emerged as the most potent compounds, with IC_{50} values of 4.2 and 13.3 nM against drug sensitive *P. falciparum* 3D7 parasites and of 6.6 and 15.3 nM against the W2 multidrug resistant line, respectively (Tables 1 and 3). Their analogues **30**-**35**, tested in the following focused screening, showed a clear reduction of potency (Table 2). Since the biochemical activity and selectivity of **30**-**35** against the tested HDAC isoforms (Table S3 in Supplementary material) were quite similar as to those of **1** and **6**, this drop of potency in 3D7 cells could be due to different cell permeability and/or pharmacokinetic properties.

Given the potential role of **1** in inhibiting HDAC activity, the effect of this compound on histone H4 acetylation in *P. falciparum* was tested. Compound **1** treatment ($3 \times IC_{50}$) caused increased H4 acetylation (up to 2-fold compared to parasites treated with vehicle (0.05% DMSO) only, data not shown), supporting the action of this compound through an inhibition of *P. falciparum* HDAC activity, although these data cannot determine if this is a direct or indirect effect. Since **1** displayed low toxicity against murine and human cell lines (SI = 186 and 763, respectively) as well as promising PK data, we examined the activity of **1** in a rodent model of malaria infection. Mice treated with **1** did not show any reduction of peripheral blood parasitemia at day 4 pi or beyond, either after oral administration or a combination of oral/iv injections. In mice treated with the antimalarial control drug chloroquine (10 mg/kg; *p.o.*) no blood stage parasites were detected (not shown). The lack of *in vivo* activity could not be linked to differences in targeted HDAC enzymes as they are well conserved between *P. falciparum* and *P. berghei* (60 up to 95% peptide sequence identity). The failure of **1** to cure mice at doses up to 50 mg/kg twice a day may be due to a too short exposure of parasites to the compound even after a 4-day treatment or a lack of exposure due to binding of **1** to plasma proteins as its free fraction was not evaluated. Another possibility may be that *P. berghei* is less sensitive to the inhibitors as

a result of functional HDAC redundancy and/or a tighter control of target expression. However, in the context of hydroxamate HDAC inhibitors, it is noteworthy that other studies have also reported high potencies *in vitro*, but poor antimalarial activities in *in vivo* murine models [19,22,85,86].

In our primary screen, among the HMT inhibitors tested, only the two G9a inhibitors **18** and **19** displayed high levels of parasite growth inhibition with IC_{50} values of 20.8 and 67.9 nM, respectively, as expected (Table 1). Nevertheless, they showed general low selectivity for the parasite versus mammalian cells (Table 4). The human DNMT3a-selective inhibitor **28** [40] showed an IC_{50} value of 325.5 nM against drug-sensitive *P. falciparum* parasites (Table 1). The tested **28** analogues revealed lower IC_{50} values than **28**, the most effective being **36**-**38**, **43** and **45** (IC_{50} values ranging between 33.6 and 10.1 nM, Table 2). This increased potency could be linked to the insertion of a methoxy group at the C6 position of the quinazoline ring (**37**, **38**, **43, 45**) and a further methoxy (**37, 38**), benzyloxy (**43**) or hydroxy (**45**) group at C7. In addition, while **43** and **45** maintained the *N*-phenylpiperazine group at quinazoline C2 and the *N*-benzylpiperidyl-4-amino moiety at quinazoline C4 position, **36**-**38** displayed a 4-fluorobenzylamino substituent at C2 and a *N*-4-methoxyphenylethylpiperidinyl-4-amino (**36**, **38**) or a *tert*-butoxycarbonylpiperidinyl-4-amino (**37**) group at C4. Importantly, **37**, **43** and **45** were also potent against the multidrug-resistant W2 line of *P. falciparum* (Table 3) and were parasite-selective inhibitors (Table 4).

To further explore the capability of these DNMT inhibitors to impact P*f*DNMT, we examined their activity using parasite extracts and a commercially available fluorimetric assay. However, as a result of very low signals in untreated samples these experiments were unable to discriminate between DNMT activities present in untreated extracts versus treated parasites (data not shown). Nevertheless, the high potency and selectivity of these compounds led us to

investigate their efficacy *in vivo*. After four or five oral doses, the parasitemia of mice treated with **37** (20 mg/kg) was still below the limit of detection on day 6 pi with a significant reduction apparent up to day 11 pi. As the PK of *p.o.* administered **43** and **45** indicated that parasiticidal plasma concentrations of these compounds can be achieved in mice, *in vivo* anti-plasmodial experiments were performed. Both compounds were found to be equally effective by oral route in mice infected with *P. berghei.* No blood-stage parasites were detected at day 4 pi in any of the mice treated with these compounds (Fig. 2D and 2E). In the case of **43**, a dose of 25 mg/kg, twice a day for four days, was sufficient to reduce parasite growth at day 4 pi to below our detection limits. For **45**, oral efficacy was observed according to the suppressive Peters 4-day test at 50 mg/kg twice a day for four days without any toxic effects. The lack of *in vivo* toxicity of **45** may be associated, at least in part, to the deletion of the benzyl group present in **43**. Regarding the absence of blood parasites up to 15 days pi, it is likely that this compound is highly active and/or potential metabolites could be produced that remain active against *P. berghei.*

In conclusion, our studies on the DNMT inhibitors **37**, **43** and **45** indicate potent activities against blood stage parasites, including multi-drug resistant parasites, and showed that these compounds, given by oral route, are highly active in animal models of malaria, contributing to the indispensable research of new antimalarial agents with new lead-like molecules.

Declarations of interest

None.

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Appendix A. Supplementary data. Experimental section. Table S1. Physical, chemical and spectral (^{1}H-NMR) data for the intermediate compounds **52-54**, **57-66**, **72-74**, **78-80**, **82**, **83**, **85**, and **86**. Table S2. Elemental analysis results for **6**, **30-32**, **34**, **35**, and **36-48**. Table S3. IC_{50} values (μM) of **6, 30-32, 34**, and **35** against HDAC1, HDAC4 and HDAC6. Table S4. DNMT1, DNMT3a and G9a inhibition by compounds **36-48**. Figure S1 (*In vivo* efficacy of **37**, **45** and **43** in *P. berghei*-infected mice). ^{1}H- and ^{13}C-NMR spectra of the reported novel final compounds **6**, **30-32**, **34**, **35**, and **36-48**.

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Highlights

- Eighteen new compounds showed potent activity against *P. falciparum*
- Pharmacokinetic studies allowed selecting of four compounds for in *in vivo* studies
- Three DNMT3a inhibitors showed oral efficacy in the murine malaria model