Article

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Shahul Hameed P.[†], *Murugan Chinnapattu*[†], *Gajanan Shanbag*[†], *Praveena Manjrekar*[†], *Krishna Koushik*[†], *Anandkumar Raichurkar*[†], *Vikas Patil*[†], *Sandesh Jatheendranath*[†] *Suresh Rudrapatna*[†], *Shubhada Barde*[†], *Nikhil Rautela*[‡], *Disha Awasthy*[‡], *Sapna Morayya*[‡], *Chandan Narayan*[‡], *Stefan Kavanagh*[Ω], *Ramanatha Saralaya*[§], *Sowmya Bharath*[§], *Pavithra Viswanath*[‡], *Kakoli Mukherjee*[‡], *Balachandra Bandodkar*[†], *Abhishek Srivastava*[§], *Vijender Panduga*[§], *Jitender Reddy*[§], *KR Prabhakar*[§], *Achyut Sinha*[‡], *María Belén Jiménez-Díaz*[±], *María Santos Martínez*[±], *Iñigo Angulo-Barturen*[±], *Santiago Ferrer*[±], *Laura María Sanz*[±], *Francisco Javier Gamo*[±], *Sandra Duffy*[¥], *Vicky M Avery*[¥], *Pamela A. Magistrado*[∞], *Amanda K. Lukens*[∞], *Dyann F. Wirth*[∞], *David Waterson*[£], *V. Balasubramanian*[†], *Pravin S. Iyer*[†], *Shridhar Narayanan*[‡], *Vinayak Hosagrahara*[§], *Vasan K. Sambandamurthy*[‡]*, *Sreekanth Ramachandran*[†]*

[†]Department of Medicinal Chemistry; [‡]Department of Biosciences and [§]DMPK and animal sciences, AstraZeneca India Pvt. Ltd., Bellary Road, Hebbal, Bangalore 560024, India; [Ω]Safety assessment, AstraZeneca, Alderley Park, Macclesfield, U.K.; [£] Medicines for Malaria Venture, International Center Cointrin, Route de Pré-Bois 20, Post Office Box 1826, 1215 Geneva, Switzerland; [¥] Discovery Biology, Eskitis Institute for Drug Discovery, Griffith University, Queensland 4111, Australia; [±] Tres Cantos Medicines Development Campus. Diseases of developing Word (DDW), GlaxoSmithKline, Severo Ochoa 2, Tres Cantos 28760, Madrid, Spain; [∞]Harvard School of Public Health, Boston, MA02115.

KEYWORDS: Malaria, cross resistance, SCID model, structure activity relationship, pharmacokinetics, efficacy

Abstract

Whole cell high throughput screening of the AstraZeneca compound library against the asexual blood stage of *Plasmodium falciparum* (*Pf*), led to the identification of amino imidazoles, a robust starting point for initiating a hit-to-lead medicinal chemistry effort. Structure-activity relationship studies followed by pharmacokinetics optimization resulted in the identification of **23** as an attractive lead with good oral bioavailability. Compound **23** was found to be efficacious (ED_{90} of 28.6 mg/kg) in the humanized *P. falciparum* mouse model of malaria (*Pf*/SCID model). Representative compounds displayed a moderate to fast killing profile that is comparable to chloroquine. This series demonstrates no cross-resistance against a panel of *Pf* strains with mutations to known antimalarial drugs, thereby suggesting a novel mechanism of action for this chemical class.

Introduction

Malaria continues to be a major global public health challenge with an estimated 627, 000 deaths in 2012.[1] The African subcontinent had the highest incidence (80%) and death toll (90%) from malaria, and most deaths (77%) occurred in children under 5 years of age.[1] Among the five distinct *Plasmodium* species that cause malaria, *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* account for more than 95% of clinical cases and deaths due to this disease. The rapid emergence and spread of malarial parasites resistant to currently available antimalarial drugs, including artemisinin derivatives, threaten to derail the global efforts to treat and prevent malaria.[2] Globally, with an estimated 3.4 billion people being at risk of contracting malaria, there is an urgent need for the discovery and development of novel and chemically distinct classes of compounds leading to effective and affordable drugs against malaria.[3]

Over the last decade, whole cell screening efforts have led to greater success in delivering new molecular entities approved by the FDA compared to classical target-based approaches.[4] A number of novel chemical entities currently under preclinical and clinical development against protozoan parasites have emerged from whole cell screening efforts.[5 - 8] In particular, phenotypic high-throughput screening campaigns have resulted in the identification of novel chemotypes active against the erythrocytic stage of *Pf* parasites, which is primarily responsible for the clinical manifestations of malaria.[9 - 12] A phenotypic high throughput screen of 500,000 compounds from the AstraZeneca corporate library, against the asexual blood stage of *Pf,* was carried out using a high content image-based approach previously reported.[13] The screening cascade began with single-point determination of growth inhibition at 1.6 μM, followed by a dose response study of actives (compounds displaying >50% growth inhibition). A hit triage exercise by applying filters for lead-like properties such as

molecular weight (<500), lipophilicity, number of hydrogen bond donors/acceptors led to the identification of several attractive starting points.

Results and Discussion

Antiplasmodial Activity and Structure−Activity Relationship (SAR). Following the hit triage, the 2-amino imidazole **1** was selected as a promising hit for structure activity exploration. This prioritized scaffold consists of a 2-amino imidazole core with an N-linked phenylethyl tether (**Fig 1**). Analysis of the activities of close analogs from the corporate collection screened revealed that the primary amine and the benzylic hydroxyl (1, 4-amino alcohol motif) groups were critical for the antimalarial activity. Hence, the initial SAR exploration in the synthetic program focused on identifying the best substituents on the phenyl ring for antimalarial potency while monitoring two selectivity readouts, i.e., cytotoxicity toward A549 mammalian cell line[14] and effect on the hERG ion channel (**Table 1**). All compounds were screened for their antimalarial activity against a sensitive (NF54) and multidrug resistant (K1) strain of *Pf* using a SYBR-Green based readout.[15,16] Chloroquine, pyrimethamine, and artesunate were included as reference drugs in all of the experiments.

A chloro substitution at R_2 position of the phenyl ring was found to be critical with 2,3-dichloro and 2,4-dichloro being the best for antiplasmodial activity of (**1 – 4 in Table 1**). On the imidazole ring, hydrophobic groups at R and R_1 were well tolerated (**5 - 8**). These compounds were not cytotoxic (displayed good selectivity index of >50), and a majority of them were weakly active against the hERG ion channel (hERG IC_{50} > 22μM).

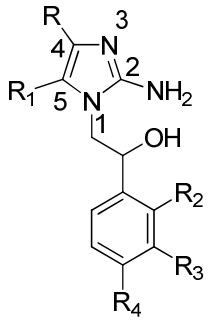


Fig 1: 2-amino imidazole

Compound	R	R_1	R_2	R_3	R_4	NF54 IC_{50} (μM)	K1 IC_{50} (μM)	Cytotoxicity index (A 549)	hERG IC_{50} (μM)
1	H	H	Cl	H	Cl	0.38	0.28	>263	ND[a]
2	H	H	F	H	Cl	>2	>2	>50	60
3	H	H	Me	H	Cl	>2	>2	>50	ND[a]
4	H	H	Cl	Cl	H	0.35	0.23	>285	22
5	Me	H	Cl	H	Cl	0.38	0.25	131	>33.3
6	Me	Me	Cl	H	Cl	0.3	0.2	220	>33.3
7	Cyclopropyl	H	Cl	H	Cl	0.12	0.07	241	4.5
8	4-Fluorophenyl	H	Cl	H	CF_3	0.14	0.06	72	25

Table 1: *In vitro* antiplasmodial activity, cytotoxicity and hERG inhibition profile of compounds **1** – **8.** Selectivity index against A549 (human lung carcinoma cell line) was calculated by dividing the *Pf* IC_{50} by the IC_{50} generated in the A549 cytotoxicity assay. ND[a]: not determined

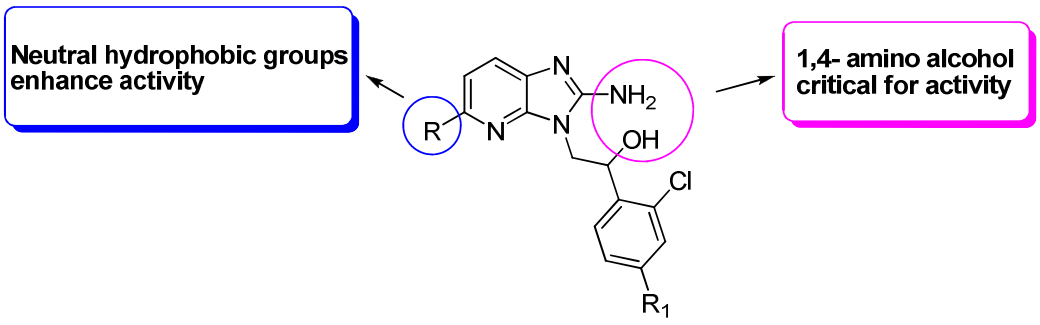
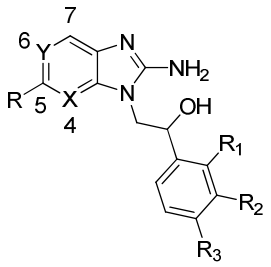


Fig 2: Structure activity features of amino substituted azabenzimidazole

Having established that 4, 5- lipophilic group substitution on the imidazole ring was tolerated, further SAR explorations were continued with the 2-amino benzimidazoles **Fig 2**. Unsubstituted 2-amino benzimidazole **9** (**Table 2**) was one of the best compounds in the series with a potency of 28 nM against the sensitive *Pf* NF54 strain. However, compound **9** had a high clearance and was rapidly metabolized in mouse microsomal and rat hepatocytes, while moderate clearance was observed in human microsomes. This is likely to be due to the hydroxylation of electron-rich benzimidazoles followed by phase II elimination. Hence, medicinal chemistry efforts were directed towards blocking the metabolic soft spots in molecules to address their high metabolic clearance.



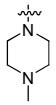
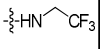
Compound	R	X	Y	R_1	R_2	R_3	NF54 IC_{50} (μM)	K1 IC_{50} (μM)	Cytotoxicity selectivity index (A549)	Rat Hep (μl/min/1E6)	Hu mics (μl/min/mg)	Mu mics (μl/min/mg)	hERG IC_{50} (μM)
9	H	C	C	Cl	H	Cl	0.028	0.007	607	155	25	213	10.3
10	CN	C	C	Cl	Cl	H	0.65	0.3	0.86	53	96	ND[a]	>33.3
11	SO_2Me	C	C	Cl	Cl	H	>2	>2	>50	44	68	ND[a]	>33.3
12	Cl	C	C	Cl	Cl	H	0.32	0.04	2.3	22	49	ND[a]	>33.3
13	CF_3	C	C	Cl	H	C_3	0.053	0.01	94	31	9	62	>10.5
14	H	C	N	Cl	H	Cl	>2	>0.5	>25	14	5	41	ND[a]
15	H	N	C	Cl	Cl	H	0.34	0.18	26	110	69	ND[a]	>33.3
16		N	C	Cl	H	CF_3	0.089	0.085	348	106	21	102	12.2
17		N	C	Cl	H	Cl	0.04	0.014	375	198	17	87	3.5
18		N	C	Cl	H	Cl	0.096	0.022	>260	94	47	>130	ND[a]
19		N	C	Cl	H	Cl	>2	>0.5	>10	ND[a]	ND[a]	ND[a]	ND[a]
20		N	C	Cl	H	CF_3	0.043	0.01	232	36	45	21	>33.3
21		N	C	Cl	H	CF_3	0.025	0.018	800	105	76	90	16.6
22		N	C	Cl	H	CF_3	>2	>0.5	>50	14	<4	ND[a]	>33.3
23		N	C	Cl	H	CF_3	0.04	0.005	>325	43	32	19	>33.3

Table 2: *In-vitro* antiplasmodial activity, cytotoxicity and hERG inhibition profile of compounds **9** – **23.** Selectivity index against A549 (human lung carcinoma cell line) was calculated by dividing the *Pf* IC_{50} by the IC_{50} generated in the A549 cytotoxicity assay.

ND[a]: not determined

The 5-substituted 2-aminobenzimidazoles **10** - **13** showed good microsomal stability with **13** being the best in terms of stability and potency. The introduction of nitrogen at 6-position of benzimidazole **14** seemed unfavorable for antiplasmodial potency, while the nitrogen at 4-

position **15** was tolerated. Since 5-substitution appeared to provide the potential for metabolic stability, other functional groups at this position were explored as follows. The N-linked compounds **16** – **17** and the C-linked compound **18** retained antiplasmodial activity whereas the amide **19** turned out to be inactive. Finally, the O-linked compounds **20** – **23** provided the balance of potency and microsomal stability with **23** being the best with adequate potency (*Pf* IC_{50} 0.04μM) and metabolic stability in different species. The compound **23** also showed good cytotoxicity selectivity index (>325-fold) and was inactive in the hERG inhibition assay (IC_{50} >33 μM).

Having developed the SAR and the essentiality of the 1,4-amino alcohol moiety for antiplasmodial activity, we undertook a limited scaffold hopping strategy to find an alternate heterocyclic core by synthesizing compounds **24** - **28** (**Table 3**). While the 2-amino triazole **24** and 5-amino tetrazole **25** were inactive, the 2-amino pyridine **26**, the phenolic linked 2-aminobenzimidazole **27** and the 2-amino quinazoline **28** retained antiplasmodial activity. These diverse heterocyclic cores could provide an alternate avenue for multiple hit to lead campaigns for these 1, 4-amino alcohol inhibitors.

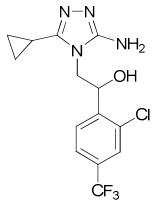
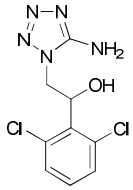
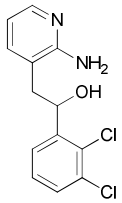
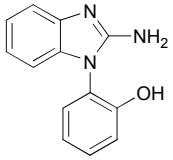
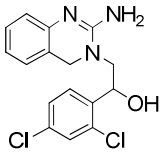
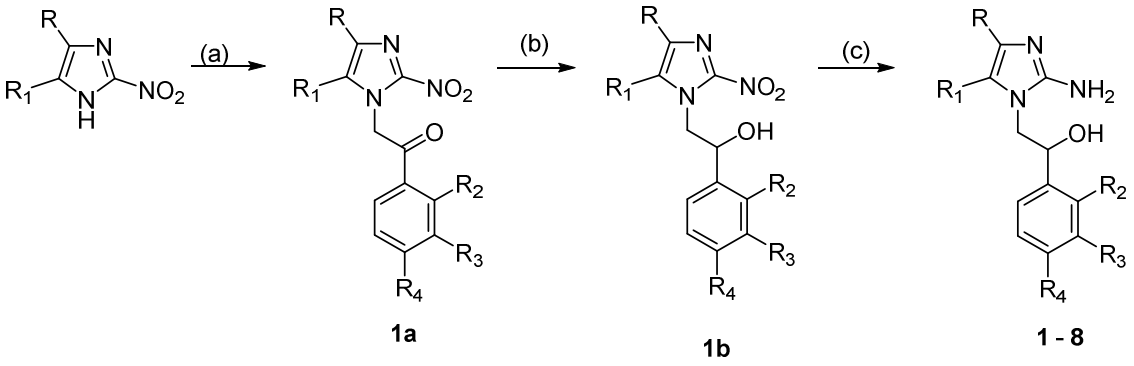
Compound	Structure	NF54 IC_{50} (μM)	K1 IC_{50} (μM)	Cytotoxicity selectivity index (A549)
24		>2	>2	>50
25		>2	>2	>50
26		0.18	0.05	294
27		0.084	0.054	1190
28		0.177	0.025	141

Table 3: Scaffold hopping of 1,4-amino alcohols to generate compounds **24** – **28** and their antiplasmodial, cytotoxicity and hERG activity. Selectivity index against A549 (human lung carcinoma cell line) was calculated by dividing the *Pf* IC_{50} by the IC_{50} generated in the A549 cytotoxicity assay.

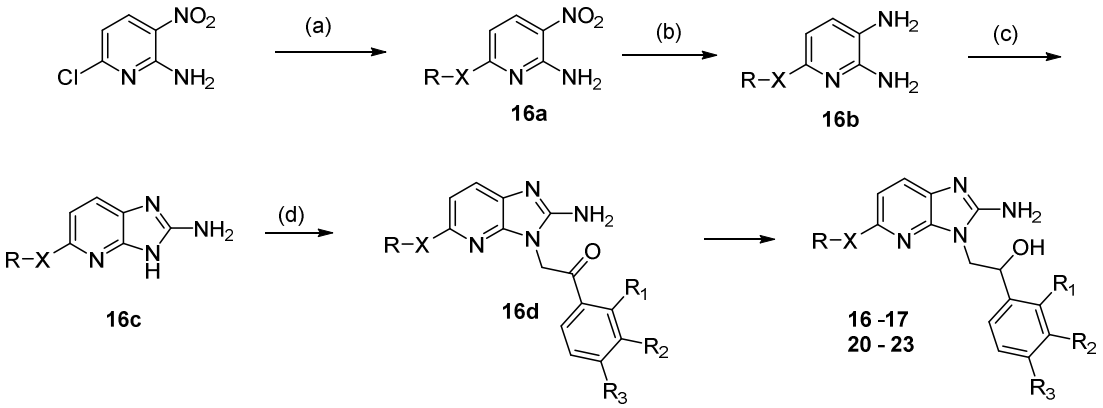
Chemistry: The 2-amino imidazoles **1** – **4** were synthesized by the base promoted N-alkylation of 2-nitro imidazoles with phenacyl bromides, followed by the sequential reduction of keto and the nitro group as depicted in Scheme **1**. Compounds **5** – **8** were synthesized by alternate routes (see supporting information).



(a) phenacyl bromide, K_2CO_3, DMF, rt, 12 h (b) $NaBH_4$, MeOH (c) Zn, NH_4Cl, MeOH, rt

Scheme 1: General synthetic scheme for 2-amino imidazoles

The general synthesis of 5-substituted-4-azabenzimidazoles **16 - 17** and **20 - 23** is shown in **Scheme 2**. Initial displacement of 6-chloro of 6-chloro-3-nitropyridin-2-amine with various alcohols and amines under basic condition yielded the 6-substituted 3-nitropyridine-2-amines **(16a)**. This was then subjected to reduction under Pd/C to provide the diamines **(16b)**, which was then cyclized to 2-amino benzimidazoles (**16c)** using cyanogen bromide. Alkylation of **16c** with phenacyl bromides using K_2CO_3 provided the N-substituted ketoamines **16d** which were reduced with $NaBH_4$ to the corresponding 1, 4-amino alcohols **16 - 17**, **20** – **23**. Compounds **9 - 15**, **18**, **19**, **24** – **28** were synthesized by alternate routes (see supporting information).



Reagents: (a) R-XH (X = O, N), NaH, THF, rt, 70% (b) Pd/C, H_2, rt, 6 h, 90% (c) CN-Br, MeOH: H_2O (1:1), 90 ^{0}C, 3 h, 60% (d) phenacyl bromide, K_2CO_3, DMF, rt, 50% (e) $NaBH_4$, MeOH, rt, 80%

Scheme 2: General scheme for the synthesis of 5-substituted-4- azabenzimidazoles

In vitro and in vivo DMPK profile. Representative compounds were profiled for various *in vitro* DMPK properties, and CYP inhibition (**Table 4**).

				CYP IC_{50} (μM)				
compound	Aq. Solubility (μM)	LogD	Hu PPB (%) free	CYP3A4	CYP2D6	CYP2C9	CYP2C19	CYP1A2
13	3	>4.6	<1	>30	26.3	27.9	2.7	29.4
15	26	3	11.8	>30	>30	>30	10.2	5.2
16	118	2.8	23.4	14.1	27.9	>30	5	>30
21	160	3.7	2.6	2.4	12.5	>30	0.78	>30
23	5	>4.5	<1	20.3	28.3	22.9	4.7	>30

Table 4: *In vitro* DMPK profile of representative compounds. Aqueous solubility, logD, estimation of free fraction in human plasma and inhibition of CYP isozymes were performed using protocols reported earlier.[17]

There appears to be an inverse correlation between logD and aqueous solubility for this series. A direct correlation between logD and extent of binding to human plasma proteins was observed. The series showed low to moderate inhibition against a human CYP P450 panel (5 isoforms) (**Table 4**).

In order to understand the pharmacokinetic behavior of this series, a study was performed in Wistar rats with compound **23** being administered orally (5 mg/kg) and *via* the intravenous route (1 mg/kg). The *in vivo* blood clearance following intravenous dosing was found to be moderate clearance (23 mL/min/kg) and a large volume of distribution (2.5 L/kg). Compound **23** showed excellent oral bioavailability (100%) (**Table 5**).

Rat PK	Compound **23**
Intravenous (1 mg/kg)	
$t_{1/2}$ (h)	1.76
AUC (μM.h)	1.66
CL (ml/min/kg)	22.6
Vss (l/Kg)	2.49
Oral (5 mg/kg)	
% F	100
$t_{1/2}$ (h)	7.16
Cmax (μM)	2.04

Table 5: Rat *in vivo* pharmacokinetic profile for compound **23.** Two rats were used for each of the dose groups. The reported values are the average PK parameters (n=2).

In vivo efficacy studies. Given the attractive antiplasmodial activity of this series against NF54 and K1 strains of *P. falciparum* along with favorable pharmacokinetic properties, *in vivo* proof of concept study in a murine *P. berghei* model of malaria[18] was undertaken with compound **23**. Following four once-daily doses of **23** at 50 mg/kg, a 94% reduction in parasitemia was observed. At this dose, **23** was not curative in this efficacy model (mean survival time of 12 ± 2 days). Chloroquine dosed at 30mg/kg for four days resulted in a 99.9% reduction in parasitemia and mice survived for 27 days (mean survival time of 27 ± 3).

This efficacy data provided the first *in vivo* proof of target engagement following oral administration of an amino azabenzimidazole in a murine model of malaria. Based on the encouraging efficacy obtained in the *P. berghei* model, we embarked on determining the therapeutic efficacy of this compound in the highly relevant humanized *Pf*/SCID efficacy model.[19] Efficacy was assessed following administration of a single oral dose per day (3, 10, 30 and 50 mg·kg^{-1}) of compound **23** for four consecutive days, and by measuring the effect on blood parasitemia by flow cytometry. Two parameters of efficacy were estimated in the study – the ED_{90} and the exposure required for achieving an ED_{90} ($AUCED_{90}$) (Figure **3a**, **3b**).

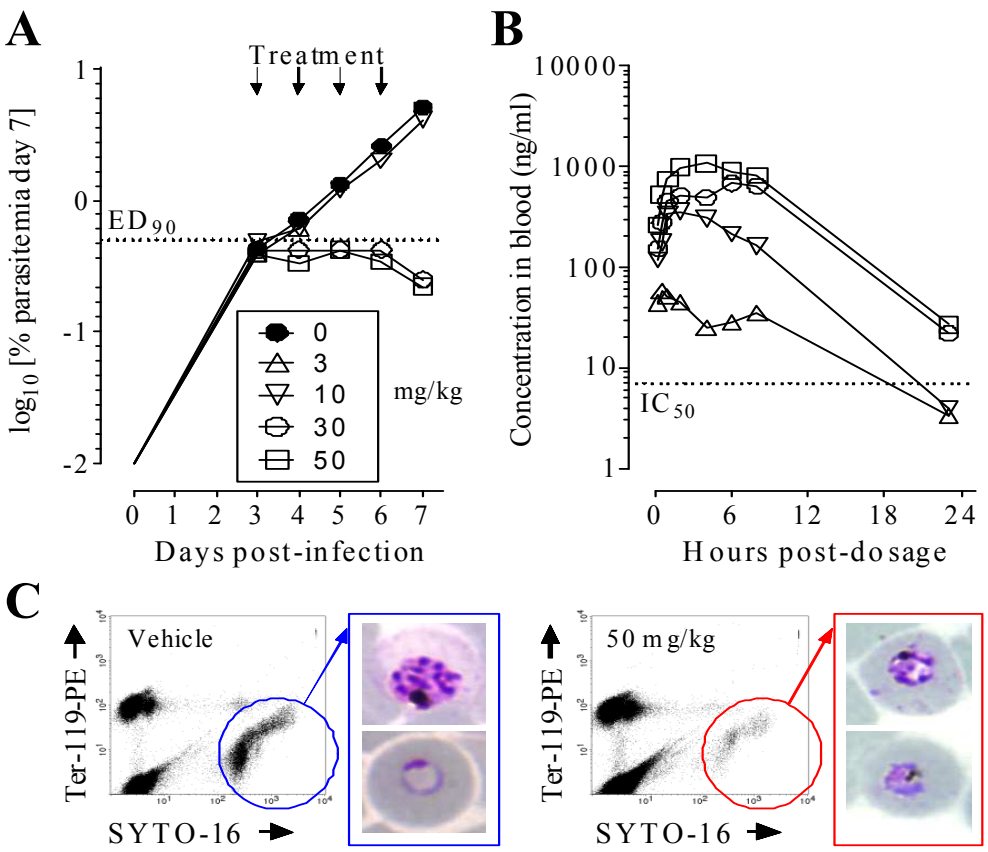


Figure 3. Efficacy of compound **23** in a humanized *P. falciparum* mouse model.

3a represents parasitemia in peripheral blood of mice infected with *P. falciparum* *Pf*3D7$^{0087/N9}$ corresponds to individual parasitemia for mice treated with **23** (n=4, where each individual was treated at different dose level) or vehicle (n=2). **3b** represents the whole blood levels of **23** after the first dose of treatment in the very same individuals of the efficacy experiment. Data represents individual profiles of blood concentration versus time of **23** (0-23 hours). The dotted line shows the *in vitro* IC_{50} in ng·ml^{-1}. **3c** represents the phenotype of parasites remaining in the peripheral blood of mice after 4 days of exposure to compound **23**. Under these experimental conditions, **23** was found to be efficacious against *Pf*, with an ED_{90} of 28.6 mg·kg^{-1} and an estimated AUC_{ED90} of 6.55 µg·h·ml^{-1}·day^{-1}.

The therapeutic efficacy of **23** against *P. falciparum* 3D7 in a '4-day test' is shown in Figure **3a**. The blood levels of **23** were measured on day 1 following oral administration of the compound to *P. falciparum*-infected NOD-SCID IL-2Rγ^{null} mice. The experimental design was optimized for analysis of efficacy parameters (ED_{90}, AUC_{ED90}). The pharmacokinetic parameters estimated following oral administration of **23** are shown in **Table 6**. The compound blood concentration versus time data profiles are plotted in Figure **3b**.

Dose (mg/kg)	C_{max} (µg/ml)	C_{max} / Dose (µg/ml per mg/kg)	t_{max} (hours)	AUC $_{(0-23)}$ (µg.h/ml)	DNAUC[a] $_{(0-23)}$ (µg.h/ml per mg/kg)
3	0.6	0.019	0.5	0.48	0.16
10	0.36	0.035	2	2.7	0.9
30	0.68	0.022	6	6.93	2.31
50	1.07	0.021	4	10.45	3.48
[a]DNAUC, dose normalised value of AUC					

Table 6. Pharmacokinetic parameters estimated in blood after oral gavage administration of **23** to *Pf*3D7$^{0087/N9}$-infected humanized SCID mice.

Non-linear fitting to a sigmoid dose-response curve of log_{10} of % parasitemia at day 7 after infection versus the dose was used to estimate the ED_{90}. Non-linear fitting to a sigmoid dose-

response curve of $\log_{10}$ of % parasitemia at day 7 after infection versus the area under the curve of levels of compound obtained during the first 23 hours after the first administration was used to estimate the potency of **23**. The potency was expressed as the daily exposure that reduced parasitemia from peripheral blood at day 7 after infection by 90% with respect to vehicle-treated mice (AUC_{ED90}) was 6.55 $\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$. This parameter of efficacy was estimated by assuming that the drug accumulation during treatment had no significant effects on parasite reduction. Microscopic examination of *Pf*3D7$^{0087/N9}$-infected mice treated with vehicle showed erythrocytes harboring healthy rings, trophozoites and mature schizonts. At day 7 post-infection, an accumulation of trophozoites were observed in mice treated with **23.** This data demonstrates the value of compound **23** in reducing the parasite burden following infection with *Pf*.

Amino alcohols retain their activity against P. falciparum strains with known mechanism of resistance*:* The SAR was optimized based on the antiplasmodial activity of compounds against the fully sensitive NF54 strain, and the chloroquine resistant K1 strain of *P. falciparum*. To rule out cross-resistance to other antimalarial agents both current and under development, representative compounds were screened against a panel of well-characterized strains of *P. falciparum* using the SYBR Green assay described earlier.[15,16]

As shown in Table 7, compounds **9**, **13**, **16**, **20**, **26**, **27** retained their IC_{50} values across six different *Pf* strains (see supporting information Table 1 for the *Pf* strain details) in contrast to antimalarial agents currently in use. Chloroquine and pyrimethamine showed a large shift to higher $IC_{50's}$ against strains K1, 7G8, W2 and DD2. Atovaquone, a clinically used antimalarial agent targeting mitochondrial metabolism showed a specific increase in IC_{50} against SB1 strain.

	IC_{50} (nM)					
Compound	**NF54**	**K1**	**7G8**	**W2**	**SB1**	**DD2**
9	18.6 ± 11.6	10.14 ± 6.06	4.09 ± 2.3	11.57 ± 0.1	42.5 ± 12	30 ± 7
13	29.2 ± 24.1	12.21 ± 9.6	3.25 ± 2.2	24.1 ± 4	121.3 ± 11	48.7 ± 6
16	141.2 ± 123.9	220.48 ± 116.43	37.88 ± 35.35	156.04 ± 92.21	219.6 ± 42	369.6 ± 45
20	96 ± 98.5	50.48 ± 39.34	12.04 ± 15.44	61.96 ± 39.5	177.8 ± 30	134.8 ± 28
26	263.1 ± 289.6	109.03 ± 85.52	29.25 ± 39.66	110.49 ± 44.82	471.9 ± 39	410.2 ± 12
27	100.3 ± 66.5	252.38 ± 174.03	66.07 ± 52.04	259.62 ± 190.81	163.6 ± 18	328.9 ± 35
Artemisinin	7.05 ± 4	6.47 ± 2	1.3 ± 0.1	6.52 ± 1	16.60 ± 5	10.26 ± 2
Chloroquine	17.09 ± 5	347.29 ± 82	163.91 ± 17	374.66 ± 228	11.81 ± 4.5	251.34 ± 9
Pyrimethamine	28.26 ± 11	8558.42 ± 1983	10041.72 ± 1261	15379.7 ± 2826	10.85 ± 4	13838.59 ± 8619
Atovaquone	1.36 ± 0.4	2.26 ± 0.8	2.32 ± 0.7	1.16 ± 0.6	>2000	2 ± 0.02

Table 7. Amino azabenzimidazoles retain their potency against a panel of *Pf* strains known to harbor mutations conferring resistance to known antimalarial agents. Data represent the IC_{50} values obtained from each compound tested in duplicate from two independent experiments.

To further eliminate overlapping mode of action with known targets, cross-resistance testing against drug resistant lines with well-characterized mutations in *Pf* dihydroorotate dehydrogenase (*Pf*DHODH)[20], *Pf* heat shock protein 90 (*Pf*HSP90) (personal communication with U.E. Ribacke), *Pf* chloroquine resistance transporter (*Pf*CRT)[21] or *Pf* cytochrome b reduction site (*Pf*CTYb-Qi) (personal communication with A.K. Lukens) was performed (see supporting information for the *Pf* strain details). Mutations in these genes contribute to resistance to antimalarial agents under development, namely 5-benzimidazolyl-N-alkylthiophene-2-carboxamide (**Genz-669178**), geldanamycin, 1,4-dibenzothiazepene amides (**IDI-3783 and IDI-5918**), respectively[21] (personal communication with U. Ribacke and A.K. Lukens). **Table 8** shows that compound **20** remained equally potent to both parental and mutant DD2 strains, in contrast to the corresponding control inhibitors (see also Supplementary **Figure 1**). This indicates that the target of compound **20** is distinct from *Pf*DHODH, *Pf*HSP90, *Pf*CRT or *Pf*CYTb-Qi. Artesunate was included as an additional control compound showing no significant difference in IC_{50s} among all of the strains tested.

Compound	IC_{50} (nM)				
	DD2	DD2-DHODH	DD2-HSP90	DD2-PfCRT	DD2-CYTbQi
20	24.6 ± 8	19.72 ± 3	34.13 ± 1	16.45 ± 1	11.79 ± 2
Artesunate	1.51 ± 0.2	1.81 ± 0.3	1.29 ± 0.2	1.84 ± 0.3	1.26 ± 0.4
29 (Genz-669178)	5.79 ± 1	**76.8 ± 10**	16.2 ± 2	5.07 ± 4	9.94 ± 0.9
Geldanamycin	176 ± 30	257 ± 40	**658 ± 30**	204 ± 20	169 ± 40
30 (ID-3783)	7.30 ± 7	16.9 ± 9	8.42 ± 1	**1045 ± 700**	3.16 ± 0.2
31 (IDI-5918)	2.60 ± 0.6	2.37 ± 0.5	ND	1.95 ± 1*	**618 ± 90**

*value reflects mean of two replicate assays ± standard deviation; ND = not determined.

Table 8. Compound **20** retains its potency against a panel of *Pf* strains known to harbor mutations conferring resistance to antimalarial agents under development. Values are the mean of three replicate assays ± standard deviation. IC_{50s} of control compounds against their specific resistant-mutant strain are highlighted in bold. *value reflects mean of two replicate assays ± standard deviation; ND = not determined.

In order to identify the mode of action of compound **20** and confirm that it is novel, a chemogenomic approach was used, whereby resistant parasites are generated by *in vitro* selection under drug pressure and whole genome sequencing is employed to identify the genetic basis of resistance[22]. However, *in vitro* resistance generation failed after multiple attempts at various parasite density and compound concentrations (see Supporting Information).

In vitro parasite reduction ratio (PRR). We employed the previously published *in vitro* PRR methodology[23] to understand the rate and extent of kill of this chemical class. Representative compounds (**20** & **21**) when tested at 10X IC_{50} displayed a fast killing profile and an effect similar to chloroquine after 48 hours of compound treatment.

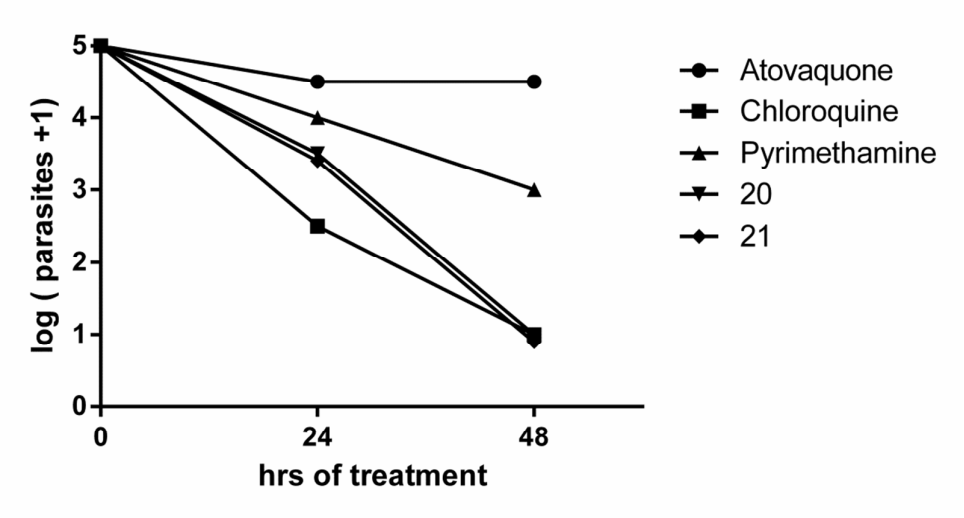


Figure 4. *In vitro* parasite reduction ratio (PRR) for compound **20** and **21**. The graph depicts the number of viable parasites following exposure of *Pf* 3D7A parasites to 10X IC_{50} concentrations of the compounds.

Most of the currently available antimalarial agents are active against the asexual blood stages of *Pf* that are primarily responsible for the clinical symptoms associated with malaria and control the emergence of drug resistance[24]. Given the global goal of eradicating malaria, newer antimalarial agents that kill the sexual forms of the parasite (transmission blocking) or target the quiescent hepatic forms of the parasite in addition to the blood-stage forms are desirable. Representative compounds from this series lacked activity against the male/female gametocytes[24] and were inactive in the *P. berghei* liver stage assay[25]. Contrary to some of the newer antimalarial agents in clinical development that target multiple life stages of *Pf*, the amino azabenzimidazoles lack activity against the liver and sexual stages of *Pf*. Given the wide spread prevalence of drug resistant forms of *Pf*, amino azabenzimidazoles owing to their novel mechanism of action and fast kill against the asexual forms could potentially be used in combination with other novel agents in clinical development to treat and eradicate malaria.

Conclusion: A detailed medicinal chemistry optimisation of a sub micromolar 2-amino imidazole screening hit has led to the identification of 2-amino azabenzimidazoles, a novel class of antiplasmodial agent, exhibiting nanomolar inhibitory activity against *Pf*. The representative compound **23** is orally bioavailable and efficacious in the humanised *Pf*/SCID model of malaria. The series displayed a fast rate of kill equivalent to chloroquine in the *in vitro* PRR assay. Representative compounds of this series retained their IC_{50} values against a panel of drug resistant strains of *Pf* suggesting a novel mechanism of action. Additionally, three novel chemotypes (compounds **24** - **28**) derived by scaffold hopping provide further avenues to diversify this class of inhibitors to build in desirable properties suitable for progression towards a candidate selection. In conclusion, the chemotype described in this study has desirable attributes for a novel antimalarial agent, such as fast kill, lack of cross resistance, low frequency of resistance emergence, excellent oral bioavailability and potential for low cost of goods based on fewer number of synthetic steps. Further studies are warranted to improve potency, solubility and evaluating in vivo safety profile in suitable preclinical models to progress this series towards candidate selection to treat malaria.

EXPERIMENTAL SECTION

All anhydrous solvents, reagent grade solvents for chromatography and starting materials were purchased from either Sigma Aldrich Chemical Co. or Fisher Scientific. Water was distilled and purified through a Milli-Q water system (Millipore Corp., Bedford, MA). General methods of purification of compounds involved the use of silica cartridges purchased from Grace Purification systems. The reactions were monitored by TLC on pre-coated Merck 60 F254 silica gel plates and visualized using UV light (254 nm). All compounds were analyzed for purity by HPLC and characterized by ^{1}H NMR using Bruker 300 MHz NMR and/or Bruker 400 MHz NMR spectrometers. Chemical shifts are reported in ppm (δ) relative to the residual solvent peak in the corresponding spectra; chloroform δ 7.26, methanol δ 3.31, DMSO-d6 δ 3.33 and coupling constants (*J*) are reported in hertz (Hz) (where s = singlet, bs = broad singlet, d = doublet, dd = double doublet, bd = broad doublet, ddd = double doublet of doublet, t = triplet, tt – triple triplet, q = quartet, m = multiplet) and analyzed using ACD NMR data processing software. Mass spectra values are reported as *m/z* (HRMS). All reactions were conducted under Nitrogen and monitored using LCMS unless otherwise noted. Solvents were removed *in vacuo* on a rotary evaporator. All the compounds exemplified in this work are either racemic mixtures or diastereomically pure compounds.

General synthetic procedures for compounds 1 - 4:

1-(2,4-Dichlorophenyl)-2-(2-nitro-1H-imidazol-1-yl)ethanone (1a) : To a stirred solution of 2-Nitro-1H-imidazole (0.5 g, 4.424 mmoles,) in acetonitrile (5 mL) phenacyl bromide (1.1 g, 4.424 mmoles) was added followed by diisopropyl ethylamine (1.1 g, 8.848 mmoles). The reaction mixture was heated under microwave conditions at 150 °C for 2 h. After confirming the absence of the starting material, acetonitrile was removed under reduced pressure. The crude residue obtained was purified by column chromatography to obtain 0.8 g (60%) of 1-(2,

4-dichlorophenyl)-2-(2-nitro-1H-imidazol-1-yl)ethanone **(1a)**. LCMS [M+1]: 301.8. ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 7.96 (dd, J = 8.00 Hz, 1H), 7.86-7.87 (m, 1H), 7.70 (m, 1H), 7.66 (m, 1H), 7.28 (m, 1H), and 6.02 (s, 2H).

1-(2,4-Dichlorophenyl)-2-(2-nitro-1H-imidazol-1-yl)ethanol (1b): To a stirred solution of **1a** (0.8 g, 2.666 mmoles) in MeOH (15 mL) sodium borohydride (0.2 g, 5.332 mmoles) was added portion wise slowly at 0 °C under N_2 atm. The reaction mixture was then stirred at room temperature for overnight. After confirming the absence of the starting material the reaction mixture was cooled and quenched with water. Then it was extracted with ethyl acetate and washed with water and brine solution. The combined organic layer was concentrated under vaccum to obtain 0.7 g (86 %) of **1b**. LCMS [M+1] : 303. ^{1}H NMR, 400 MHz, DMSO-d6: δ ppm 7.58 - 7.57 (m, 1H), 7.46-7.38 (m, 3H), 7.11 (d, J = 4.00 Hz, 1H), 6.09 (d, J = 8.00 Hz, 1H), 5.26-5.21 (m, 1H), and 4.59-4.57 (m, 2H).

2-(2-Amino-1H-imidazol-1-yl)-1-(2,4-dichlorophenyl)ethanol (1) : To a stirred solution of **1b** (0.7 g, 2.3 mmol) in MeOH (10 mL) activated zinc powder (1 g, 16.22 mmol) and ammonium chloride (1.2 g, 23 mmol) were successively added. The resulting mixture was stirred at room temperature for overnight. After confirming the absence of the starting material the reaction mixture was filtered through celite and washed with MeOH. The organic layer was concentrated and purified by column chromatography to obtain 433 mg (69%) of 2-(2-amino-1H-imidazol-1-yl)-1-(2,4-dichlorophenyl)ethanol **1**. HRMS for $C_{11}H_{11}Cl_2N_3O$ $[M+H]^+$: 272.03541. ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 8.27 (s, 1H), 7.62-7.60 (m, 2H), 7.50-7.48 (m, 1H), 7.10 (s, 2H), 6.68 (s, 1H), 6.63-6.62 (m, 1H), 5.16 – 5.13 (m, 1H), and 3.96-3.94 (m, 2H). HPLC 98.91 %.

2-(2-Amino-1H-imidazol-1-yl)-1-(4-chloro-2-fluorophenyl)ethan-1-ol (2) : Compound **2** was prepared using similar synthetic scheme as described above for compound 1 . HRMS for

$C_{11}H_{11}ClFN_3O$ $[M+H]^+$: 256.06496. ^{1}H-NMR (400 MHz, DMSO-d6): δ ppm 7.56-7.58 (m, 3H), 7.40-7.43 (m, 1H), 7.32-7.35 (m, 1H), 6.82-6.86 (m, 2H), 6.18 (s, 1H), 5.10-5.13 (m, 1H), 4.16-4.17 (m, 1H), and 4.12-4.14 (m, 1H). HPLC 99.72 %.

2-(2-Amino-1H-imidazol-1-yl)-1-(4-chloro-2-methylphenyl)ethan-1-ol (3): Compound **3** was prepared using similar synthetic scheme as described above for for compound 1. HRMS for $C_{12}H_{14}ClN_3O$ $[M+H]^+$: 252.08925; ^{1}H NMR **(**400 MHz, DMSO-d_6) δ ppm 7.45-7.52 (m, 3H), 7.26-7.28 (m, 2H), 6.89-6.90 (m, 2H), 5.88 (d, *J* = 4.36 Hz, 1H), 5.00-5.01 (m, 1H), 3.94-4.05 (m, 2H), and 2.33 (s, 3H). HPLC 93.59 %.

2-(2-Amino-1H-imidazol-1-yl)-1-(2,3-dichlorophenyl)ethanol (4): Compound **4** was prepared using similar synthetic scheme as described above for compound 1. HRMS for $C_{11}H_{11}Cl_2N_3O$ $[M+H]^+$: 272.0353. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 3.59-3.83 (m, 1H), 3.83-3.97 (m, 1H), 5.15 (m, 3H), 6.11 (d, *J*=4.33 Hz, 1H), 6.32 (d, *J*=1.32 Hz, 1H), 6.43 (d, *J*=1.32 Hz,1H), 7.32-7.46 (m, 1H), 7.48-7.67 (m, 2H). HPLC purity: 98.74%.

2-(2-Amino-4-methyl-1H-imidazol-1-yl)-1-(2,4-dichlorophenyl)ethanol (5): Compound **5** was prepared by an alternate procedure (see supporting information). HRMS for $C_{12}H_{13}Cl_2N_3O$ $[M+H]^+$: 286.05079. ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 7.60 (d, J = 8.00 Hz, 1H), 7.49 (d, J = 2.00 Hz, 1H), 7.39 (dd, J = 2.00, 6.60 Hz, 1H), 6.36 (s, 1H), 5.30-5.31 (m, 1H), 3.99 (m, 2H) and 2.09 (s, 3H). HPLC: 99.04%.

2-(2-Amino-4,5-dimethyl-1H-imidazol-1-yl)-1-(2,4-dichlorophenyl)-ethan-1-ol (6): Compound **6** was prepared by an alternate scheme (see supporting information). HRMS for $C_{13}H_{15}Cl_2N_3O$ $[M+H]^+$: 300.06686. ^{1}H-NMR (400 MHz, MeOD): δ ppm 7.58 (d, J = 8.40 Hz, 1H), 7.48 (d, J = 2.04 Hz, 1H), 7.36-7.38 (m, 1H), 5.27-5.30 (m, 1H), 3.97 (d, J = 2.92 Hz, 1H), 3.93 (d, J = 2.96 Hz, 1H), 1.94 (s, 3H), and 1.82 (s, 3H). HPLC purity: 99.63%.

2-(2-Amino-4-cyclopropyl-1H-imidazol-1-yl)-1-(2,4-dichlorophenyl)ethanol (7): Compound **7** was prepared using an alternate scheme (see supporting information) HRMS for $C_{14}H_{15}Cl_2N_3O$ $[M+H]^+$: 312.06302. ^{1}H NMR **(**400 MHz, CD_3OD) δ ppm 7.60 (d, J = 8.40 Hz, 1H), 7.49 (d, J = 1.92 Hz, 1H), 7.39 (dd, J = 1.88, 8.40 Hz, 1H), 6.34 (s, 1H), 5.33 (t, J = 5.20 Hz, 1H), 3.96-4.02 (m, 2H), 1.68-1.72 (m, 1H), 0.89-0.93 (m, 2H), and 0.59-0.62 (m, 2H). HPLC purity: 98 %.

2-(2-Amino-4-(4-fluorophenyl)-1H-imidazol-1-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (8) : Compound **8** was prepared by an alternate scheme (see supporting information). HRMS for $C_{18}H_{14}ClF_4N_3O$ $[M+H]^+$: 400.08366. ^{1}H NMR **(**400 MHz, DMSO-d_6) δ ppm 7.7-7.86 (m, 3H), 7.58-7.61 (m, 2H), 7.06-7.10 (m, 2H), 6.96 (s, 1H), 6.19-6.20 (d, 1H), 5.37 (s, 2H), 5.22 (s, 1H), 3.92-3.97 (m, 1H), and 3.79-3.85 (m, 1H). HPLC purity 93.76 %.

Compounds **9** – **15** were synthesized by alternate routes. The detailed synthetic scheme for each compound is provided in the supporting information.

2-(2-Amino-1H-benzo[d]imidazol-1-yl)-1-(2,4-dichlorophenyl)ethanol (9) : HRMS for $C_{15}H_{13}Cl_2N_3O$ $[M+H]^+$: 322.05057. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 3.96 - 4.19 (m, 2 H) 5.14 - 5.31 (m, 1 H) 6.04 - 6.11 (m, 1 H) 6.12 - 6.31 (m, 1 H) 6.75 - 6.88 (m, 1 H) 6.88 - 6.96 (m, 1 H) 6.95 - 6.96 (m, 1 H) 6.99 (d, *J*=7.54 Hz, 1 H) 7.06 - 7.18 (m, 1 H) 7.40 - 7.51 (m, 1 H) 7.56 - 7.63 (m, 1 H) 7.68 - 7.78 (m, 1 H). ES+MS m/z: 322.30 (M+1).

2-Amino-1-(2-(2,3-dichlorophenyl)-2-hydroxyethyl)-1H-benzo[d]imidazole-6-carbonitrile (10): HRMS from $C_{16}H_{12}Cl_2N_4O$ $[M+H]^+$: 347.04688. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 4.25 (d, 2 H) 5.25 - 5.39 (m, 1 H) 6.02 - 6.19 (m, 1 H) 6. 81 (s, 2 H) 7.20 - 7.35 (m, 1 H) 7.36 – 7.40 (m, 2 H) 7.45 - 7.50 (m, 1 H) 7.61 - 7.63 (m, 1 H) 7.75 - 7.79 (m, 1 H). HPLC purity: 99%.

2-(2-Amino-6-(methylsulfonyl)-1H-benzo[d]imidazol-1-yl)-1-(2,3-dichlorophenyl)ethanol (11) : HRMS for $C_{16}H_{15}ClN_3O_3S$ $[M+H]^+$: 400.02941. ^1H NMR (300 MHz, DMSO-d6) δ ppm 3.06 (s, 3H), 4.24 (d, *J*=5.65 Hz, 2H), 5.32 (q, *J*=5.46 Hz, 1H), 6.11 (d, *J*=4.52 Hz, 1H), 6.80 (s, 2H), 7.25 (d, *J*=8.29 Hz, 1H), 7.35-7.53 (m, 3H), 7.58 (dd, *J*=7.91, 1.32 Hz, 1H), 7.62-7.74 (m, 1H). HPLC purity: 99.29%

2-(2-Amino-6-chloro-1H-benzo[d]imidazol-1-yl)-1-(2,3-dichlorophenyl)ethanol (12) : HRMS for $C_{15}H_{12}Cl_3N_3O$ $[M+H]^+$: 356.00879. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 4.05 - 4.23 (m, 2 H) 5.18 - 5.36 (m, 1 H) 6.04 - 6.16 (m, 1 H) 6.29 - 6.49 (m, 2 H) 6.85 - 6.98 (m, 2 H) 7.01 - 7.14 (m, 1 H) 7.33 - 7.45 (m, 1 H) 7.52 - 7.61 (m, 1 H) 7.62 - 7.70 (m, 1 H). HPLC purity: 99%.

2-(2-Amino-6-(trifluoromethyl)-1H-benzo[d]imidazol-1-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (13): HRMS for $C_{17}H_{12}ClF_6N_3O$ $[M+H]^+$: 424.06424. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 4.17 - 4.38 (m, 2 H) 5.29 - 5.40 (m, 1 H) 6.12 - 6.22 (m, 1 H) 6.62 - 6.80 (m, 2 H) 6.91 - 7.03 (m, 1 H) 7.12 - 7.25 (m, 2 H) 7.62 - 7.79 (m, 2 H) 7.81 - 7.91 (m, 1 H). HPLC purity: 97 %.

2-(2-Amino-1H-imidazo[4,5-c]pyridin-1-yl)-1-(2,4-dichlorophenyl)ethanol (14): HRMS for $C_{14}H_{12}Cl_2N_4O$ $[M+H]^+$: 323.04563. ^1H NMR (300 MHz, DMSO-*d*6) δ ppm 4.09 - 4.22 (m, 2 H) 5.15 - 5.32 (m, 1 H) 6.08 (d, *J*=4.52 Hz, 1 H) 6.57 (s, 2 H) 6.97 (d, *J*=5.27 Hz, 1 H),7.46 (dd, *J*=8.38, 2.17 Hz, 1 H) 7.57 (d, *J*=2.07 Hz, 1 H) 7.69 (d, *J*=8.48 Hz, 1 H) 7.97 (d, *J*=5.27 Hz, 1 H) 8.34 (s, 1 H). HPLC purity: 99%.

2-(2-Amino-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2,3-dichlorophenyl)ethanol (15): HRMS for $C_{14}H_{12}Cl_2N_4O$ $[M+H]^+$: 323.04731. ^1H NMR (300 MHz, DMSO-d6) δ ppm 4.15-4.32 (m, 2H), 5.45 (m, 1H), 6.20 (s, 1H), 6.62 (bs, 2H), 6.92 (m,1H), 7.35 (m, 2H), 7.55 (m, 1H), 7.65 (m, 1H), 7.80 (m, 1H). HPLC purity: 99.17%.

General synthetic procedures for compounds 16 – 17, 20 - 23:

2-(2-Amino-5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethan-1-ol (16):

6-(4-Methylpiperazin-1-yl)-3-nitropyridin-2-amine (16a):

To the solution of 6-chloro-3-nitropyridin-2-amine (2.0 g, 17.0 mmol) in anhydrous DMF (20 mL) were added 1-methyl piperazine (3.46 g, 50.70 mmol) and potassium carbonate (4.77 g, 50 mmol). The resulting mixture was heated at 50°C for 16 h. The reaction mixture was poured in to ice cold water and solid precipitated was filtered and dried to obtain 6-(4-methylpiperazin-1-yl)-3-nitropyridin-2-amine as a yellow solid. Yield: 1.6 g (59.25%). LCMS: m/z 238.4 $(M+H)^+$. ^{1}H NMR 300 MHz, DMSO-d6: δ ppm 8.02-8.05 (m, 3H), 6.33 (d, J = 9.54 Hz, 1H), 3.69 (s, 4H), 2.34 (t, J = 4.98 Hz, 4H), and 2.19 (s, 3H).

6-(4-Methylpiperazin-1-yl)pyridine-2,3-diamine (16b): To a solution of 6-(4-methylpiperazin-1-yl)-3-nitropyridin-2-amine (1.6 g, 6.74 mmol) in methanol : DCM (50 mL) was added palladium (10%) on carbon (0.5 g). The mixture was stirred under hydrogen atmosphere for 1 h. The suspension was filtered through celite and filtrate was concentrated under reduced pressure to obtain 6-(4-methylpiperazin-1-yl)pyridine-2,3-diamine which was taken to next step without further purification. Yield: 0.8 g (57.5%). LCMS: m/z 208.7 $(M+H)^+$.

5-(4-Methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-2-amine (16c): To the solution of 6-(4-methylpiperazin-1-yl)pyridine-2,3-diamine (0.54 g, 2.60 mmol) in MeOH : H_2O (1 : 1) (50mL) was added cyanogen bromide (0.551 g, 5.21 mmol) at 0°C. The dark green mixture was stirred at RT for 16 h. The solvent was evaporated to dryness and the residue was triturated with acetonitrile to obtain brown residue which was purified by column

chromatography using 10% MeOH in DCM saturated with ammonia as eluent to obtain 5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-2-amine as brown solid. LCMS: m/z 233.4 $(M+H)^+$. ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 10.62 (s, 1H), 7.21 (d, J = 8.36 Hz, 1H), 6.29 (d, J = 8.36 Hz, 1H), 6.15 (s, 2H), 3.30 (t, J = 4.88 Hz, 4H), 2.41 (t, J = 4.80 Hz, 4H), and 2.21 (s, 3H).

2-(2-Amino-5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethan-1-one (16d) : To the solution of 5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-2-amine (0.171 g, 0.73 mmol) in acetonitrile (5 mL) was added 2-bromo-1-(2-chloro-4-(trifluoromethyl)phenyl)ethan-1-one (0.221 g, 0.73 mmol) and DIPEA (0.243 mL, 1.47 mmol). The resulting mixture was subjected to microwave irradiation in a sealed tube at 150°C for 2 h. The product formation was confirmed by LCMS. The solvent was evaporated to dryness and the residue obtained was purified by column chromatography using 5% MeOH in DCM saturated with ammonia as eluent to obtain 2-(2-amino-5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethan-1-one as off white solid. Yield: 0.048 g (14.44%). ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 8.14 (d, J = 8.04 Hz, 1H), 8.08 (s, 1H), 7.98 (d, J = 8.04 Hz, 1H), 7.32 (d, J = 8.36 Hz, 1H), 6.45 (t, J = 8.48 Hz, 3H), 5.41 (s, 2H), 3.32 (t, J = 5.32 Hz, 4H), 2.38 (t, J = 4.80 Hz, 4H), and 2.20 (s, 3H).

2-(2-Amino-5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethan-1-ol (16): To the solution of 2-(2-amino-5-(4-methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4(trifluoromethyl)phenyl) ethan-1-one (0.043 g, 0.094 mmol) in methanol (1 mL) was added sodium borohydride (0.0036 g, 0.094 mmol) at 0°C. The mixture was stirred at RT for 1 h. The reaction mixture was quenched with EtOAc and evaporated to dryness and the residue was purified by column chromatography using 4% MeOH in DCM as eluent to obtain 2-(2-amino-5-(4-

methylpiperazin-1-yl)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4(trifluoromethyl)phenyl) ethan-1-one as off white solid. Yield: 0.013 g (37.20%). HRMS for $C_{20}H_{22}ClF_3N_6O$ $[M+H]^+$: 455.15743. ^{1}H NMR 400 MHz, CD_3OD: δ ppm 7.66 (t, J = 8.28 Hz, 2H), 7.44 (d, J = 7.96 Hz, 1H), 7.33 (d, J = 8.48 Hz, 1H), 6.44 (d, J = 8.52 Hz, 1H), 5.58 (t, J = 5.12 Hz, 1H), 4.33-4.45 (m, 2H), 3.41 (s, 4H), 2.60 (t, J = 4.96 Hz, 4H), and 2.39 (s, 3H). HPLC 97.31 %.

2-(2-Amino-5-(2,2,2-trifluoroethylamino)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2,4-dichlorophenyl)ethanol (17) : Compound **17** was prepared using similar synthetic scheme as described above for compound **16**. HRMS for $C_{16}H_{14}Cl_2F_3N_5O$ $[M+H]^+$: 420.06022. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 3.88 - 4.26 (m, 4 H) 5.28 - 5.43 (m, 1 H) 6.02 - 6.12 (m, 2 H) 6.13 - 6.19 (m, 1 H) 6.19 - 6.26 (m, 1 H) 6.55 - 6.70 (m, 1 H) 7.14 - 7.28 (m, 1 H) 7.38 - 7.47 (m, 1 H) 7.54 - 7.60 (m, 1 H) 7.63 - 7.71 (m, 1 H). HPLC 98.5 %.

Compounds **18** and **19** were synthesized by alternate routes. The detailed synthetic scheme for each compound is provided in the supporting information

2-(2-Amino-5-cyclopropyl-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2,4-dichlorophenyl)ethanol (18): HRMS for $C_{17}H_{16}Cl_2N_4O$ $[M+H]^+$: 363.07717. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 7.63 (d, *J* = 8.48 Hz, 1H), 7.54 (d, *J* = 2.07 Hz, 1H), 7.41 (dd, *J* = 2.07, 8.48 Hz, 1H), 7.18 - 7.28 (m, 1H), 6.85 (d, *J* = 7.72 Hz, 1H), 6.46 (s, 2H), 6.11 (d, *J* = 4.52 Hz, 1H), 5.35 (dd, *J* = 3.96, 7.91 Hz, 1H), 3.97 - 4.23 (m, 2H), 1.96 (t, *J* = 6.03 Hz, 1H), 0.72 - 0.90 (m, 4H). HPLC purity: 95.3 %.

2-Amino-N-cyclopropyl-3-(2-(2,4-dichlorophenyl)-2-hydroxyethyl)-3H-imidazo[4,5-b]pyridine-5-carboxamide (19): HRMS for $C_{18}H_{17}Cl_2N_5O_2$ $[M+H]^+$: 406.08353. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 0.48 - 0.66 (m, 2 H) 0.72 - 0.86 (m, 2 H) 2.75 - 2.91 (m, 1 H) 4.15 - 4.39 (m, 2 H) 5.31 - 5.47 (m, 2 H) 6.41 - 7.07 (bs,2 H) 7.31 - 7.46 (m, 2 H) 7.47 - 7.56 (m, 1 H) 7.58 - 7.72 (m, 2 H) 7.76 - 7.96 (m, 1 H). HPLC purity: 98 %.

2-(2-Amino-5-(2,2,2-trifluoroethoxy)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (20): Compound **20** was prepared using similar synthetic scheme as described above for compound **16**. HRMS for $C_{17}H_{13}ClF_6N_4O_2$ $[M+H]^+$: 455.07107. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 4.02-4.31 (m, 2H), 4.67-4.91 (m, 2H), 5.47 (d, *J*=4.33 Hz, 1H), 6.26 (d, *J*=4.33 Hz, 1H), 6.35-6.52 (m, 3H), 7.42 (d, *J*=8.10 Hz, 1H), 7.67 (d, *J*=9.04 Hz, 1H), 7.72-7.86 (m, 2H). HPLC purity: 96.95%.

2-(2-Amino-5-(tetrahydrofuran-3-yloxy)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (21): Compound **21** was prepared using similar synthetic scheme as described above for compound **16**. HRMS for $C_{19}H_{18}ClF_3N_4O_3$ $[M+H]^+$: 443.1089. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.57 - 7.88 (m, 3H), 7.35 (dd, *J* = 1.51, 8.10 Hz, 1H), 6.21 - 6.45 (m, 3H), 5.47 (br. s., 1H), 5.10 - 5.29 (m, 1H), 4.10 - 4.30 (m, 2H), 3.59 - 3.94 (m, 5H), 2.03 - 2.26 (m, 1H), 1.87 - 2.02 (m, 1H). HPLC purity: 95.5%.

2-(2-Amino-5-(azetidin-3-yloxy)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (22) : Compound **22** was prepared using similar synthetic scheme as described above for compound **16**. HRMS for $C_{18}H_{17}ClF_3N_5O_2$. HCl $[M+H]^+$: 428.10923. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 1.89 (s, 3H), 3.40-3.60 (m, 2H), 3.68-3.84 (m, 2H), 3.99-4.30 (m, 2H), 5.07 (m, 1H), 5.45 (t, *J*=5.37 Hz, 1H), 6.21-6.40 (m, 3H), 7.36 (d, *J*=8.29 Hz, 1H), 7.60-7.75 (m, 1H), 7.76-7.88 (m, 2H). HPLC purity: 99.45%.

2-(2-Amino-5-(1,1,1-trifluoropropan-2-yloxy)-3H-imidazo[4,5-b]pyridin-3-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl)ethanol (23): Compound **23** was prepared using similar synthetic scheme as described above for compound **16**. HRMS for $C_{18}H_{15}ClF_6N_4O_2$ $[M+H]^+$: 469.08154. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.39 (d, *J*=6.59 Hz, 3 H) 4.15 - 4.24 (m, 2 H) 5.41 - 5.51 (m, 1 H) 5.58 - 5.70 (m, 1 H) 6.25 - 6.32 (m, 1 H) 6.39 - 6.44 (m, 1 H) 6.45 -

6.52 (m, 2 H) 7.35 - 7.48 (m, 1 H) 7.67 - 7.76 (m, 1 H) 7.80 - 7.89 (m, 2 H). HPLC purity: 99 %.

Compounds **24** – **28** were synthesized by alternate routes. The detailed synthetic scheme for each compound is provided in the supporting information.

2-(3-Amino-5-cyclopropyl-4H-1,2,4-triazol-4-yl)-1-(2-chloro-4-(trifluoromethyl)phenyl) ethanol (24): HRMS for $C_{14}H_{14}ClF_3N_4O$ $[M+H]^+$: 347.08817. ^{1}H NMR 400 MHz, DMSO-d6: δ ppm 7.88-7.91 (m, 2H), 7.80-7.82 (m, 1H), 5.33-5.34 (m, 2H), 4.11-4.24 (m, 3H), 2.12-2.18 (m, 1H), 0.97-1.10 (m, 3H), and 0.90 (m, 2H). HPLC purity 99.17 %.

2-(5-Amino-1H-tetrazol-1-yl)-1-(2,6-dichlorophenyl)ethanol (25) : HRMS for $C_9H_9Cl_2N_5O$ $[M+H]^+$: 274.02504. ^{1}H NMR 400 MHz, CD_3OD: δ ppm 7.40 (d, J = 8.00 Hz, 2H), 7.27 (t, J = 8.36 Hz, 1H), 6.04 (q, J = 5.64 Hz, 1H), 5.13 (dd, J = 8.28, 13.62 Hz, 1H), and 4.81 (dd, J = 5.64, 13.60 Hz, 1H). HPLC purity 92.62 %.

2-(2-Aminopyridin-3-yl)-1-(2,3-dichlorophenyl)ethanol (26): HRMS for $C_{13}H_{12}Cl_2N_2O$ $[M+H]^+$: 283.03962. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 2.56-2.80 (m, 2H), 5.20 (m, 1H), 5.67 (s, 2H), 5.72-5.96 (m, 1H), 6.47 (dd, *J*=7.25, 4.99 Hz, 1H), 7.15 (d, *J*=5.84 Hz, 1H), 7.29-7.46 (m, 1H), 7.55 (dd, *J*=7.91, 1.32 Hz, 1H), 7.58-7.67 (m, 1H), 7.81 (dd, *J*=4.90, 1.70 Hz, 1H). HPLC purity: 98 %.

2-(2-Amino-1H-benzo[d]imidazol-1-yl)phenol (27): HRMS for $C_{13}H_{11}N_3O$ $[M+H]^+$: 226.09711. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 5.99 (br. s., 2H), 6.56-6.70 (m, 1H), 6.81 (t, *J*=7.44 Hz, 1H), 6.87-7.03 (m, 2H), 7.10 (d, *J*=8.29 Hz, 1H), 7.14-7.29 (m, 2H), 7.29-7.46 (m, 1H), 9.89-10.13 (m, 1H). HPLC purity: 99.3 %.

2-(2-Aminoquinazolin-3(4H)-yl)-1-(2,4-dichlorophenyl)ethanol (28): HRMS for $C_{16}H_{15}Cl_2N_3O$ $[M+H]^+$: 336.06636. ^{1}H NMR **(**400 MHz, CD_3OD) δ ppm 7.74 (d, *J* = 8.44

Hz, 1H), 7.48-7.49 (m, 1H), 7.41 (dd, *J* = 2.04, 8.40 Hz, 1H), 7.28-7.32 (m, 1H), 7.13-7.15 (m, 2H), 7.01 (d, *J* = 7.92 Hz, 1H), 5.43-5.46 (m, 1H), 4.68 (d, *J* = 14.48 Hz, 1H), 4.45 (d, *J* = 14.52 Hz, 1H), 3.87-3.93 (m, 1H), and 3.63-3.67 (m, 1H). HPLC 99.66 %.

Measuring *in vitro* antiplasmodial activity. Compounds were tested in duplicate on two independent occasions against chloroquine-sensitive NF54 (MRA-1000, MR4, ATCC, Manassas, Virginia) and chloroquine-resistant K1 strains of *Pf*. A modified method of Trager and Jensen was employed to maintain continuous *in vitro* cultures of asexual blood stages of *Pf*.[26] Quantitative assessment of antiplasmodial activity *in vitro* was determined following 72 hours of compound exposure using the SYBR I method as described earlier.[15] The percent inhibition with respect to the drug-free control was plotted against the logarithm of drug concentration. The growth inhibition curves were fitted by non-linear regression using the sigmoidal dose–response (variable slope) formula to yield the concentration–response curves. The IC_{50} value of the compound was defined as the lowest concentration at which 50% inhibition was observed. Chloroquine diphosphate (CQ) (Sigma), artesunate (Sigma), and pyrimethamine were used as reference drugs in all experiments.

Plasmodium falciparum strain details. The following strains used for cross-resistance testing, DD2-PfCRT and DD2-CYTbQi were generous gifts from A.K. Lukens, DD2-DHODH was a generous gift from L.S. Ross and DD2-HSP90 was a generous gift from U. Ribacke. These mutant strains were generated elsewhere by in vitro drug resistance selection in the presence of IDI-3783 to generate DD2-PfCRT (Lukens et al, 2013), IDI-5918 to generate DD2-CYTbQi (personal communication with A.K. Lukens), Genz-669178 and GSK3 to generate DD2-DHODH (Ross et al, 2014) and geldanamycin to generate DD2-HSP90 (personal communication with U. Ribacke). Mutations in the respective target genes, Plasmodium falciparum chloroquine resistance transporter (PfCRT) and Pf heat shock protein 90 (PfHSP90) were confirmed by whole genome sequencing while Pf cytochrome b

reduction site (PfCTYb-Qi) and Pf dihydroorotate dehydrogenase (PfDHODH) were confirmed by PCR and Sanger sequencing (Ross et al, 2014, Lukens et al, 2013 and personal communication with U. Ribacke and A.K. Lukens).

Cross-resistance testing. The following strains used for cross-resistance testing, DD2-*Pf*CRT and DD2-CYTbQi were generous gifts from A.K. Lukens. DD2-DHODH was a generous gift from L.S. Ross and DD2-HSP90 was a generous gift from U. Ribacke. These mutant strains were generated elsewhere by in vitro drug resistance selection in the presence of compound **30** to generate DD2-*Pf*CRT[21], compound **31** to generate DD2-CYTbQi (personal communication with A.K. Lukens), Compound **29** to generate DD2-DHODH[20] and geldanamycin to generate DD2-HSP90 (personal communication with U. Ribacke). Mutations in the respective target genes, *Plasmodium falciparum* chloroquine resistance transporter (*Pf*CRT) and *Pf* heat shock protein 90 (*Pf*HSP90) were confirmed by whole genome sequencing, while *Pf* cytochrome b reduction site (*Pf*CTYb-Qi) and *Pf* dihydroorotate dehydrogenase (*Pf*DHODH) were confirmed by PCR and Sanger sequencing [20,21] and personal communication (U. Ribacke and A.K. Lukens).

A549 cytotoxicity assay. The *in vitro* cytotoxicity of compounds were measured against A549 human lung carcinoma cells as described previously.[14] Briefly, A549 cells (ATCC) were grown in RPMI medium (GIBCO BRL) containing 10% heat-inactivated fetal bovine serum (GIBCO-BRL) and 1mM L-glutamine (GIBCO-BRL) at a density of ~1,000 cells/well. After incubation of the cells with compound in a CO_2 atmosphere at 37°C for 72 hours, cell viability was determined following addition of 10μM of resazurin solution (Sigma), by measuring fluorescence (excitation at 535 nm, emission at 590 nm) using a fluorimeter. The concentration at which growth is inhibited by 50% is determined as IC_{50} value.

***In vitro* ADME and pharmacokinetic studies.** All studies were performed as described earlier.[17]

***In vivo P. berghei* efficacy model**. Compounds with promising *in vitro* antiplasmodial activity along with an acceptable toxicity/pharmacokinetic profile were tested for in vivo efficacy. A standard 4-day suppression test[18] using the rodent malaria parasite *P. berghei* (GFP-ANKA strain) was employed to assess the efficacy of a compound by tracking the reduction in % parasitemia following 4 daily oral dosing. All animal experimentation protocols were approved by the Institutional Animal Ethics Committee registered with the Government of India (Registration No: 5/1999/CPCSEA). Adult male BALB/c mice (purchased from RCC laboratories, Hyderabad, India) were used for efficacy studies. Animals were randomly distributed to cages quarantined for one week with veterinary examination and then taken into experimentation. Feed and water were given ad libitum. Briefly, male BALB/c mice were infected intra-peritoneally with $2x10^7$ infected erythrocytes on day 0. Test compounds were administered orally in a volume of 10 mL/kg every 24 hours for 4 days starting on day 0. Two hours after the 4^{th} dose, percent parasitemia was estimated by measuring the number of viable parasites using flow cytometry (BD FACS Calibur). The effect of a test compound on parasite growth was calculated as the difference between the mean value of the control group (taken as 100%) and those of the experimental group and expressed as percent reduction in the fluorescence signal. Reference anti-malarial compound chloroquine was used as positive control and the results obtained matched those published in the literature. Pharmacokinetics analysis was performed in healthy as well as infected mice. Data from healthy mice were used for designing the dosing regimen for the efficacy studies (data not shown).

***P. falciparum* humanized mouse efficacy model.** The therapeutic efficacy of **23** against *P. falciparum* $Pf3D7^{0087/N9}$ was studied using a '4-day test' as described earlier.[19] Briefly,

NODscidIL2Rγ^{null} mice engrafted with human erythrocytes were infected with 20×10^6 *P. falciparum*-infected erythrocytes. Infections were performed by intravenous inoculation. All mice were randomly assigned to their corresponding treatment. The treatment started at day 3 and finished at day 6 after infection. In all cases, parasitemia was assessed in samples from peripheral blood obtained at days 3, 4, 5, 6, and 7 after infection.

A qualitative analysis of the effect of treatment on *P. falciparum Pf*3D7$^{0087/N9}$ was assessed by microscopy and flow cytometry. Fresh samples of peripheral blood from *P. falciparum*-infected mice were stained with TER-119-Phycoerythrine (marker of murine erythrocytes) and SYTO-16 (nucleic acid dye) and then analyzed by flow cytometry (FACS Calibur, BD). Microscopy analysis was performed with Giemsa-stained blood smears from samples taken at days 5 and 7 (48 and 96 h after starting treatment, respectively).

Pharmacokinetic analysis. The levels of **23** were evaluated in whole blood in order to determine standard pharmacokinetic parameters in the individual animals from the efficacy study. Peripheral blood samples (30 µL) were taken at different times, mixed with 120 µL of deionized water and immediately frozen on dry ice. The frozen samples were stored at -80ºC until analysis. For LC-MS/MS analysis, thawed blood samples (75µL) were processed by protein precipitation with acetonitrile (75µL) containing internal standard (carbamazepine, 10 ng/mL), extracted in a plate shaker for 10 minutes at 650 rpm and centrifuged (4000 rpm for 20 minutes at 4°C). The supernatant was separated (100µL), pipette mixed with 0.2M zinc sulphate (3µL) and centrifuged (4000 rpm for 20 minutes at 4°C). The resulting supernatant mixed with mobile phase (50% v/v) was analyzed by LC-MS/MS. A standard curve ranging from 0.002 to 20 µg/mL was used to quantitate the concentrations of **23** in whole blood. Blood concentration versus time was analyzed by non-compartmental analysis (NCA) using Phoenix vers.6.3 (from Pharsight), from which exposure-related values (C_{max} and AUC_{0-t}) and t_{max}, were estimated.

***In vitro* parasite reduction ratio (PRR) assay.** This assay was performed with the standard laboratory adapted *P. falciparum* 3D7A strain (chloroquine and pyrimethamine sensitive), as described earlier.[23] The rate of killing of a compound can be represented as the number of viable parasites (in $\log_{10}$) in function of treatment time (**Figure 1**). The PRR is defined as the log-linear reduction of viable parasites over 48 hours. Some compounds display a lag phase, defined as the time during which the number of viable parasites stays above 10^4. Based on the killing rate profile, parasite clearance time (PCT), i.e., the time to kill 99.9% of the initial population can also be determined.

ASSOCIATED CONTENT

Details of the synthesis of all compounds, and *Pf* drug resistant strains are provided as supporting information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: vasan2005@gmail.com ; ramachandran.sreekanth@gmail.com

Phone: +91-80-23621212, Fax: +91-80-23621214. The authors declare no competing financial interest.

Author Contributions

S.H, and S.R. were responsible for medicinal chemistry design and analyses. S.R., C.M., G.S., P.M., K.K., and V.P. were responsible for synthetic chemistry. S.J. performed the 2D NMR experiments and S.R. performed the HRMS analysis. S.B. was responsible for dispensing the compounds for various biochemical and DMPK assays. N.R., D.A., S.M., C.N., K.M., P.V., S.B., V.K.S., was responsible for biological profiling of compounds in various assays. S.D. and V.M.A., were responsible for the high throughput screening of the AstraZeneca library, B.J. and I.A.-B. were responsible for evaluating the efficacy parameters in the *Pf*/SCID model, M.S.M. and S.F. were responsible for the PK analysis in the *Pf*/SCID model, L.M.S. and F.J.G were responsible for the kill rates in the *in vitro* PRR assay. P.A.M, A.K.L and D.F.W were responsible for generating cross resistance and mutant selection data. V.R. and S.R. wrote the manuscript with contributions from all co-authors.

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ABBREVIATIONS

Pf, *Plasmodium falciparum*; SCID, severe combined immunodeficiency; SAR, structure-activity relationship; PRR, parasite reduction ratio; PK, pharmacokinetics

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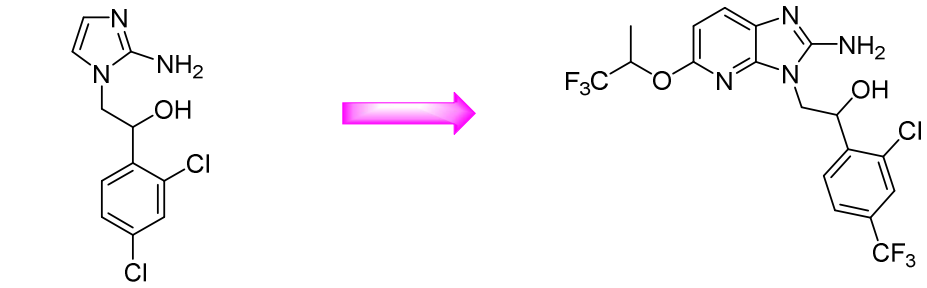
Table of Content Graphic:



HIT
Pf IC_{50}: 0.38 μM

LEAD
Pf IC_{50}: 0.04 μM
ED_{90}: 28.6 mg/kg