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Exploring a Tetrahydroquinoline Antimalarial Hit from the Medicines for Malaria Pathogen Box and Identification of its Mode of Resistance as *Pf*eEF2

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New antimalarial treatments with novel mechanism of action are needed to tackle *Plasmodium falciparum* infections that are resistant to first-line therapeutics. Here we report the exploration of MMV692140 (**2**) from the Pathogen Box, a collection of 400 compounds that was made available by Medicines for Malaria Venture (MMV) in 2015. Compound **2** was profiled in *in vitro* models of malaria and was found to be active against multiple life-cycle stages of *Plasmodium* parasites. The mode of resistance, and putatively its mode of action, was identified as *Plasmodium falciparum* translation elongation factor 2 (*Pf*eEF2), which is responsible for the GTP-dependent translocation of the ribosome along mRNA. The compound maintains activity against a series of drug-resistant parasite strains. The structural motif of the tetrahydroquinoline (**2**) was explored in a chemistry program with its structure-activity relationships examined, resulting in the identification of an analog with 30-fold improvement of antimalarial asexual blood stage potency.

Introduction

Malaria remains one of the most significant global health issues today with an estimated 241 million cases and over 627,000 deaths in 2020, the vast majority in sub-Saharan Africa.[1] This parasitic disease affects mostly children below the age of five. Pregnancy-associated malaria is also of enormous impact: about 12 million expectant mothers were exposed to malaria

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infections in 2020, resulting in the delivery of more than 800,000 children with low birthweight.[1] Of the five malaria parasite species known to infect humans, *Plasmodium falciparum* is the most prevalent species in Africa and the most virulent, responsible for the majority of both infections and deaths globally.[1] Outside Africa, another *Plasmodium* species, *Plasmodium vivax* is the most prevalent. While this species is less virulent than *P. falciparum*, it is capable of undergoing sporogonic development in the mosquito at lower temperatures and can enter into dormancy in the liver for years in the form of hypnozoites. The current standard of care treatment for malaria are fixed dose artemisinin combination therapies.[1] Though these medicines are effective and safe, partial resistance to artemisinin derivatives and frank resistance to certain other antimalarials are now spreading throughout parasite populations worldwide. There is therefore, an urgent need to develop novel therapeutics, especially those with a novel mechanism of action.[2]

The Pathogen Box is a collection of 400 compounds selected from various drug discovery programs for their potential against neglected tropical diseases and made available to the research community by Medicines for Malaria Venture (MMV) in 2015. About one third of the collection is active against *P. falciparum* parasites, while the remaining compounds show activity against other pathogens and parasites.[3] MMV009135 (**1**), a tetrahydroisoquinoline from the Pathogen Box, was identified in an open access phenotypic screening program of over 5 million compounds against *P. falciparum* (Figure 1). However, upon resynthesis of compound **1**, the IC_{50} in the 72-hour SYBR green model of blood stage malaria on the 3D7 strain was 23 μM, significantly lower than the reported potency by two different laboratories with an IC_{50} in range of 1 μM in 3D7 strain assays.[4,5] As this difference could not be explained by variation in the 3D7 assays run by different laboratories according to our experience of the assay, we investigated structurally related analogues of compound **1**. The tetrahydroquinoline MMV692140 (**2**) was identified showing restored activity with an of IC_{50} 1.8 μM in the 3D7 assay. This is an activity in range of what was previously attributed to compound **1**.

Herein we report the profile of compound **2** in pharmacological assays and the identification of its mechanism of resistance and putatively its mode of action. Further, structural analogs of this motif were synthesized and the structure activity relation (SAR) was studied in the 72 hour SYBR green 3D7 strain assay. This led to identification of MMV1919557 (**36**), an analog with significantly improved biological activity.

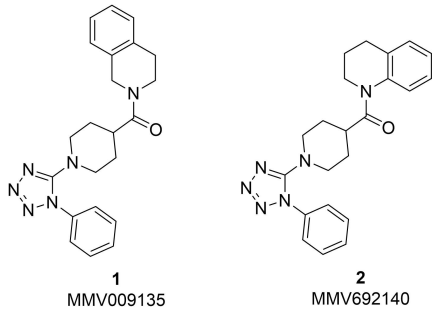


Figure 1. Structures of MMV009135 (**1**) and MMV692140 (**2**).

Results and Discussion

Pharmacological profiling of **2** showed that the compound also had activity against liver and sexual parasite lifecycle stages, in addition to its asexual blood stage activity (Table 1).

To further characterize this chemotype, the time dependent *in vitro* killing rate of **2** was determined in a parasite reduction ratio (PRR) assay. This assay uses a limiting dilution technique to quantify the number of *P. falciparum* parasites that remain viable after drug treatment over time.[6] Data are depicted in Figure 2 with comparison to antimalarial reference drugs covering a range of killing kinetics. Compound **2** was shown to be cidal with a lag phase of 48 hours and a slow killing profile.

Table 1. Profile of MMV692140 (**2**).[a,b]

P. falciparum 3D7, IC_{50}[c]	1.8 μM
P. falciparum NF54, IC_{50}[c]	0.6 μM
Liver stage, *P. berghei*, IC_{50}	0.09 μM
Sexual stage, GamV, IC_{50}	2.0 μM
Parasite reduction ratio[6]	Slow rate
Mam. Cytotoxicity, Hep G2, IC_{50}	40 μM
hERG, IC_{50}	10.6 μM
Hu Mics/Mouse Mics	>578/>578 μL/min/mg
CaCo2, (A to B/B to A)	17.5/26 10^{-6} cm/sec
Mouse PPB[d]	10% free
Log D	3.3
Solubility[e]	7 μM
Rat PK, Cl/F[f]	32.7 mL/min/Kg/<1%

[a] See Supporting Information for assay details. [b] Values are in range of MMV Validated Hit criteria; asexual blood stage *Pf* activity of 1.0 μM, mammalian cytotoxicity >10-fold window, Log D <5, MW <500, HBD <5, HBA <10, solubility >10 μM and other *in vitro* properties measured.[7] [c] Values reported are means of at least two independent experiments. [d] Binding to mice plasma protein determined by equilibrium dialysis. [e] Thermodynamic solubility of crystalline compound in phosphate buffer pH 7.4 at 25 °C for 2 h. [f] Blood PK after *i.v.* and *p.o.* administration.

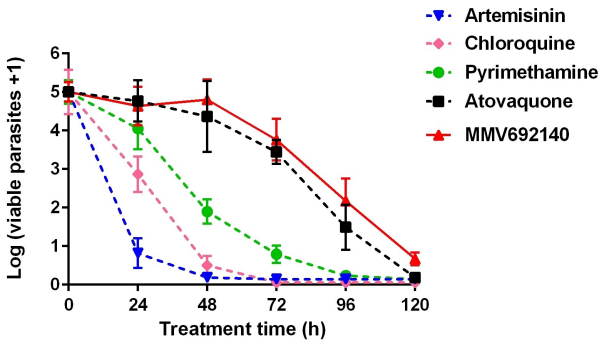
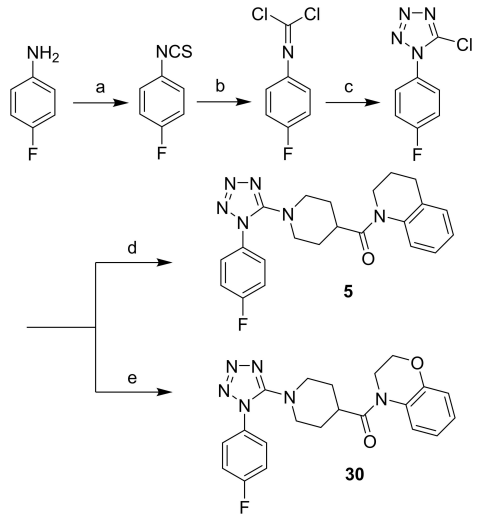


Figure 2. Parasite reduction assay. Parasites were treated with MMV692140 (**2**) at 10 x IC_{50} in the 3D7 strain and the number of viable parasites were quantified in one experiment with four technical replicates (±SD). Data from artemisinin, chloroquine, pyrimethamine and atovaquone were retrieved from previously generated data and shown for comparative purposes (dashed lines).

Metabolic *in vitro* clearance of compound **2** was significant in human and mice microsomes, and fast metabolism was also observed in *in vivo* PK studies in rat. These results were not surprising considering that the compound contains positions exposed to metabolic oxidation, such as the benzylic methylene group in the tetrahydroquinoline moiety.

The lead compound **2** was suitable for structural expansion and to further explore this series, analogs were synthesized and assessed for their blood stage antimalarial activity using the 72-hour 3D7 strain *in vitro* assay.[8] A study of the activity of modification at the aryl and tetrahydroquinoline portion of the molecule was initiated using a synthetic route exemplified by the entries **5** and **30** in Scheme 1, where variation on left- and right-side building blocks are connected via the piperidine-4-carbonyl moiety. (See Chemistry Section in Supporting Information).



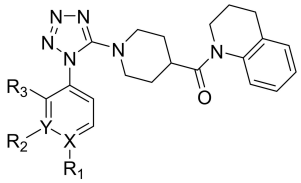
Scheme 1. General pathway for the synthesis of analogs **3**–**36**: (a) TEA, CH_2Cl_2, 25 °C, CS_2, 0–25 °C, 20 h, then T3P, EtOAc, 0–25 °C, 3 h, 60%; (b) Cl_2, CCl_4, 0–25 °C, 18 h; (c) NaN_3, TBAB, toluene, water, 25 °C, 3 h, 46% over 2 steps; (d) 1-(piperidine-4-carbonyl)-1,2,3,4-tetrahydroquinoline, *n*-BuOH, DIPEA, 130 °C, 16 h, 57%; (e) 4-(piperidine-4-carbonyl)-3,4-dihydro-2H-1,4-benzoxazine, *n*-BuOH, DIPEA, 130 °C, 16 h, 24%

Compounds were tested for *P. falciparum* blood stage activity in the 72-hour 3D7 assay. The SAR for compounds with variations in the tetrazole-phenyl moiety was relatively flat and there was no significant effect on lipophilicity of substituents (Table 2). However, electronic effects of phenyl substituents were observed. Electron donating groups, such as methoxy and methyl in the *para* or *ortho* positions improved potency (entries **7** and **8**) while electron withdrawing groups such as trifluoromethoxy, nitrile and trifluoromethoxy decreased potency (entries **9**–**11**), though effect was not large. Replacing the phenyl group with isopropyl or cyclic alkyl groups reduced activity (entries **27**–**29** in Table 4).

With regard to variations of the tetrahydroquinoline group, the SAR was generally more sensitive to modifications. Electron donating groups, such as methoxy in the 7-tetrahydroquinoline position (Table 3, R_1) were accepted (entry **16**), while electron withdrawing groups (entries **22** and **23**) were not tolerated. Substitution in the 6-tetrahydroquinoline position (Table 3, R_2) was not allowed (entries **19**–**21**) and led to inactive compounds. Substitution in the 5-tetrahydroquinoline position (Table 3, R_3) with a methoxy group led to decreased activity (entry **26**) while substitution with a chloro-substituent (entry **17**) increased the activity but also the lipophilicity, from log D 3.3 to 3.6 as compared to compound **2**.

The importance of the benzylic α-methylene position in the tetrahydroquinoline moiety for metabolism was illustrated by the preparation of the 3,4-dihydro-2H-1,4-benzoxazine derivative **18**. While all other analogs in this set (entries **3**–**26**) had a human microsome metabolism intrinsic clearance value of >100 μL/min/mg, compound **18** was the first with a clearance below 100 μL/min/mg (98 μL/min/mg), confirming the assumption that the α-methylene position is subjected to oxidative

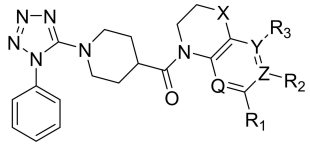
Table 2. Activity of compounds **3**–**14** against *P. falciparum* 3D7 strain.[a]



Cpd	X	Y	R_1	R_2	R_3	3D7 IC_{50} [μM]
3	N	C	–	H	H	3.7 ± 0.07
4	C	N	H	–	H	2.4 ± 0.30
5	C	C	F	H	H	4.3 ± 0.69
6	C	C	H	Cl	H	3.4 ± 0.98
7	C	C	OCH_3	H	H	0.9 ± 0.14
8	C	C	H	H	CH_3	1.4 ± 0.02
9	C	C	OCF_3	H	H	5.5 ± 0.86
10	C	C	CN	H	H	3.2 ± 0.23
11	C	C	H	H	CF_3	4.3 ± 0.08
12	C	C	H	CN	H	1.7 ± 0.06
13	C	C	H	H	Cl	1.1 ± 0.02
14	C	C	Cl	H	H	2.0 ± 0.12

[a] Values reported are geometric mean ± SEM of at least two independent experiments.

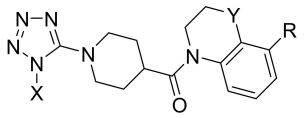
Table 3. Activity of compounds **15**–**26** against *P. falciparum* 3D7 strain.[a]



Cpd	X	Y	Z	Q	R_1	R_2	R_3	3D7 IC_{50} [μM]
15	C	C	C	N	H	H	H	4.4 ± 0.59
16	C	C	C	C	OCH_3	H	H	1.2 ± 1.2
17	C	C	C	C	H	H	Cl	0.6 ± 0.09
18	O	C	C	C	H	H	H	6.8 ± 0.75
19	C	C	C	C	H	CN	H	>10
20	C	C	C	C	H	Cl	H	>25
21	C	C	C	C	H	OCH_3	H	>25
22	C	C	C	C	CN	H	H	>25
23	C	C	C	C	Cl	H	H	>25
24	C	N	C	C	H	H	–	10.2 ± 0.75
25	C	C	N	C	H	–	H	10.8 ± 0.65
26	C	C	C	C	H	H	OCH_3	>10.0

[a] Values reported are geometric mean ± SEM of at least two independent experiments.

Table 4. Activity of compounds **3–14** against *P. falciparum* 3D7 strain.[a]



Cpd	X	Y	R	Hu Mics.[b]	3D7 IC_{50} [μM]
27	2-Propyl	C	H	135	5.6 ± 0.92
28	Cyclopentyl	C	H	>400	3.3 ± 0.48
29	Cyclohexyl	C	H	>400	3.5 ± 0.02
30	4-Fluorophenyl	O	H	83	8.9 ± 1.4
31	4-Methoxyphenyl	C	Cl	>400	0.7 ± 0.10
32	4-Methoxyphenyl	O	Cl	78	3.6 ± 1.2
33	4-Pyrrolidinylphenyl	C	Cl	>400	0.11 ± 0.01
34	4-Pyrrolidinylphenyl	O	Cl	296	1.6 ± 0.081
35	4-Pyrrolidinylphenyl	C	H	>400	0.15 ± 0.006
36	4-Pyrrolidinylphenyl	C	F	>400	0.066 ± 0.008

[a] Values reported are geometric mean ± SEM of at least two independent experiments. [b] (μL/min/mg).

Table 5. Mean IC_{50} values for clones selected for resistance to MMV692140 (**2**) and M5717.[a]

Clones	IC_{50} [μM]	Fold shift	Codon change	Amino acid, Position and Mutation
MMV692140 (2)				
Dd2-B2 (parent)	0.45			
A3	37.4	83x	TAT ->TgT	Y719C
D3	7.6	17x	TTC ->TTa	F770L
G12	18.6	41x	TTA ->TcA	L507S
M5717				
Dd2-B2 (parent)	0.0002			
D6	3.1	14700x	TAT->aAT	Y186N
E5	0.0058	27x	GAA->GAc	E134D
F8	0.66	3100x	TTA->TTt	L775F

[a] IC_{50} values represent the mean of three independent 72 hour concentration-response assays with technical duplicates. Mutations were observed in 100% of the reads for each mutant clone. M5717-resistant clones were previously reported.[9]

metabolism in this series. Still, microsomal metabolism remained relatively high for compound **18**, suggesting other parts of the molecule are subjected to metabolism as well.

A set of compounds that incorporated variations in both the phenyl and tetrahydroquinoline moieties was then generated. Again 3,4-dihydro-2H-1,4-benzoxazine derivatives showed improved metabolic stability over their tetrahydroquinoline congeners as demonstrated in matched pairs **7** and **32** with human microsome metabolism intrinsic clearance values of 521 and 78 μL/min/mg respectively. Introduction of an *N*-pyrrolidine group in the *para*-position in the phenyl group led to a significant increase in potency. The pyrrolidine **36** was the most potent one with an IC_{50} of 0.066 μM in the 72-hour 3D7 assay.

To determine the molecular target for MMV692140 (**2**), 1×10^9 *P. falciparum* asexual blood stage parasites (using the chloroquine-resistant Dd2-B2 clone) were cultured in triplicate under three times the IC_{50} of 0.72 μM as determined in 72-hour growth assays. Resistant parasites recrudesced 17 days after the selection was initiated. Parasites were then cloned by limiting dilution under constant drug pressure. Clones showed 17 to 83-fold increases in IC_{50} values compared with Dd2-B2. Genomic DNA was extracted from 3 resistant clones (from two separate flasks) that showed the highest IC_{50} shifts, and then subjected to whole-genome sequencing using 2×300 bp paired end reads and an Illumina MiSeq sequencer. The average depth of sequence coverage was 48 to 64 fold across each genome (Table S1). Analysis revealed a distinct single nucleotide polymorphism (SNP) in the elongation factor 2 gene (*Pfeef2*, PF3D7_1451100) in each of the three sequenced clones (A3, D3, and G12). These clones had acquired the *Pf*eEf2 point mutation Y719C, F770L, and L507S, respectively (Supporting Information Table 5). These clones each also harbored a second SNP, each occurring in a separate gene, which we attribute to spontaneous mutations occurring during parasite replication during the expansion of the drug-resistant parasites (Table S2). Phenotypic analysis of A3, D3 and G12 clones, along with their resistant parent, in 72 hour drug susceptibility assays with compound **2** yielded mean IC_{50} values that were 83, 17 and 41 fold higher than the Dd2-B2 parental clone, respectively (Table 5).

While *Pf*eEF2 mutations have previously been found in selections using the antimalarial compound M5717 (also known as DDD107498), which is currently being evaluated in clinical studies for the treatment of malaria, the 3 SNPs reported here are novel.[9] Y719C and F770L map to domain V and L507S to domain III of *Pf*eEF2. Drug susceptibility profiling showed that the compound **2**-selected clones A3 and G12 were not cross-resistant with M5717 (Figure 3). Clone D3 showed a nominally significant IC_{50} increase that was 1.6-fold higher than parental Dd2-B2.

We also profiled three clones that had earlier been selected for resistance to M5717.[9] These clones D6, E5 and F8, express the *Pf*eEF2 mutations Y186N, E134D and L755F, respectively, with clones D6 and F8 mediating very high-grade resistance contrasting with moderate to low resistance in the E5 clone, relative to parental Dd2-B2 (Table 5). Against compound **2**,

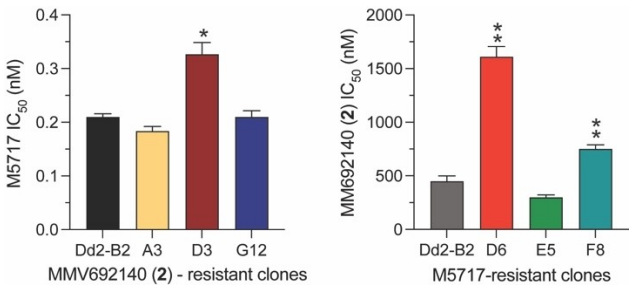


Figure 3. Cross resistance experiments using M5717 and MMV692140 (**2**). Data show mean ± SEM IC_{50} values for M5717 and MMV692140 (**2**), produced from three independent assays with technical duplicates. Unpaired *t* tests with Welch's correction were used to test for statistically significant differences between each MMV692140- or M5717-selected clone (harboring *Pf*eEF2 mutations listed above) and the parental clone Dd2-B2. *$p < 0.05$; **$p < 0.01$.

Table 6. *In vitro* intraerythrocytic antimalarial activity of **2** against field adapted strains (chloroquine and artesunate as controls).[a]

Field adapted strain	Mutated loci	Compound **2**, IC_{50} [μM][b] (fold shift)	Chloroquine IC_{50} [μM][b]	Artesunate IC_{50} [μM][b]
NF54		0.55 (−)	0.008	0.004
K1	*Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps	0.62 (1.1)	0.20	0.002
7G8	*Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps	0.47 (0.8)	0.08	0.002
TM90 C2b	*Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps, *Pf*cytb$_{Q0}$	0.72 (1.3)	0.14	0.005
Cam3.I	*Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps, *Pf*kelch13	0.79 (1.4)	0.18	0.011
Dd2	*Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps	0.90 (1.6)	0.16	0.006

[a] 72-hour [^{3}H]hypoxanthine incorporation assay. [b] mean values from two independent biological replicates.

Table 7. *In vitro* intraerythrocytic antimalarial activity of **2** against laboratory selected *P. falciparum* resistant strains.[a]

Lab adapted strains (mutated loci)	Compound **2**, IC_{50} [μM][b] (fold shift)	Ref. compounds, Dd2 (wt)/lab adapted strains (IC_{50} [nM][b] (fold shift)
Dd2 (wt)	0.895 (−)	
Dd2 (*Pf*eEF2)[9]	1.64 (1.8)	2/3100 (1550)[c]
Dd2 (*Pf*pi4k)[12]	0.778 (0.9)	21.2/102 (4.8)[d]
Dd2 (*Pf*dhodh)[13]	0.699 (0.8)	8.0/143 (18) [e]
Dd2 (*Pf*carl)[5]	0.718 (0.8)	41/2992 (73) [f]
Dd2 (*Pf*cytb)[14]	0.707 (0.8)	19/254 (13) [g]

[a] Genetically engineered *P. falciparum* strains. Mutations were introduced in the Dd2 wild-type strain. [b] Values reported are means of at least two independent experiments. Reference compounds; [c] M5717. [d] MMV390048. [e] DSM265. [f] GNF153. [g] ELQ300.

clones D6 and F8 also showed significantly higher IC_{50} values, although at a far lower level with only 3.6- and 1.7-fold increases relative to Dd2-B2. E5 showed a trend towards a lower IC_{50} compared with D2-B2, although this did not attain significance ($p = 0.07$).

However, it should be pointed out that the identification of *Pf*eEF2 as mode of resistance for compound **2** does not preclude other mechanisms being involved in cellular *Plasmodium* activity for this series.

Because minimizing the risk of resistance is critical when developing novel antimalarials, existing resistant strains must be evaluated in order to determine risk for cross resistance in parasites that are already prevalent in the field. To assess this risk, compound **2** was tested against various laboratory resistant *P. falciparum* strains to ensure activity remained constant across strains.[10] Encouragingly, full activity was maintained against all resistant lines, irrespective of their genetic background including geographical origin and resistance profile using the sensitive parasite strain NF54 as reference wild type strain, (Table 6).

Additionally, no significant cross-resistance was observed when **2** was assessed against parasites which are resistant to antimalarial agents currently being developed.[11] Though, resistant Dd2 clones adapted to withstand *Pf*eEF2 inhibition induced by M5717 showed a modest 1.8 fold shift in potency when compared to the shift observed with M5717 (Table 7). This might be explained by the different SNPs in *PfeEF2* observed with compound **2**.[8]

Conclusion

We have explored the biological, physical chemical and DMPK profile of the tetrahydroquinoline **2**. The mechanism of resistance and, putatively, its mode of action was identified as *Plasmodium falciparum* translation elongation factor 2 (*Pf*eEF2). Moreover, antimalarial killing profile determined in PRR assay is nearly identical to that determined for M5717.[9] This result provides additional evidence of *Pf*eEF2 as the parasite target for this chemical series. Nonetheless, we cannot rule out possible additional mode of action. Structural analogs of hit compound **2** were synthesized and the SAR were studied in the 72-hour 3D7 strain assay. This led to identification of MMV1919557 (**36**), an analog with 30-fold improvement of antimalarial asexual blood stage *in vitro* potency that can provide a tool to further understand the potential of *Pf*eEF2 as target for the treatment of malaria.

Experimental Section

See the Supporting Information for synthesis and characterization of new compounds and biological and DMPK assay protocols.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Malaria · *Pf*eEF2 · malaria resistance · malaria rate of killing · biological activity · drug discovery

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