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Original article

Discovery of new thienopyrimidinone derivatives displaying antimalarial properties toward both erythrocytic and hepatic stages of *Plasmodium*

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ABSTRACT

A preliminary *in vitro* screening of compounds belonging to various chemical families from our library revealed the thieno[3,2-*d*]pyrimidin-4(3*H*)-one scaffold displayed a promising profile against *Plasmodium falciparum*. Then, 120 new derivatives were synthesized and evaluated *in vitro*; compared to drug references, 40 showed good activity toward chloroquine sensitive (IC_{50} 35–344 nM) and resistant (IC_{50} 45–800 nM) *P. falciparum* strains. They were neither cytotoxic (CC_{50} 15–50 μM) toward HepG2 and CHO cells, nor mutagenic. Structure–activity relationships were defined. The lead-compound also appeared active against the *Plasmodium* liver stages (*Plasmodium yoelii* IC_{50} = 35 nM) and a preliminary *in vivo* evaluation indicated the *in vitro* activity was preserved (45% reduction in parasitemia compared to untreated infected mice). A mechanistic study demonstrated these molecules do not involve any of the pathways described for commercial drugs and exert a specific activity on the ring and trophozoite stages.

1. Introduction

Malaria is a devastating pathology, which in 2012 affected 207 million people worldwide (range 135–287 million) and caused 627,000 deaths (range 473,000–789,000) [1]. This infection, transmitted *via* the bite of the female *Anopheles* mosquito, is caused by five species of protozoan parasites belonging to the *Plasmodium* genus, namely *falciparum*, *malariae*, *vivax*, *ovale* and *knowlesi*. *Plasmodium falciparum* is the most virulent [2], causing more than 95% of malaria-related morbidity and mortality. According to the WHO [1], important and durable progress has been recorded in recent years, with the estimated incidence of malaria globally

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reduced by 25% since 2000. Moreover, malaria-specific mortality rates were also reduced by 42% between 2000 and 2012. These encouraging statistics indicate that malaria programs are having a good impact, the combined result of vector control, chemoprevention, diagnostic testing and effective malaria treatment. Nonetheless, the growing drug resistance of parasites around the world [3] remains a real and ever-present danger, attributable mainly to *P. falciparum*. Currently, the only fully effective antimalarial drugs utilize artemisinin and its derivatives in combination with several different partner drugs in artemisinin-based combination therapies (ACTs), now recommended as the first line of treatment in endemic areas. However, reduced susceptibility of *P. falciparum* to treatment with artemisinin derivatives identified and confirmed on the Cambodia–Thailand border in 2009 [4,5]. This emerging resistance could lead to a resurgence of more virulent levels of malaria unless new chemical classes of effective drugs be rapidly found. In this context, all innovative practices [6] and encouraging results are being sponsored and shared [7,8], so as to accelerate the development and licensing of new antimalarial drugs. Particularly, as the currently available 8-aminoquinolines (primaquine, tafenoquine) can lead to severe side effects such as acute intravascular hemolysis in individuals with severe glucose-6-phosphate deficiency, the search for original compounds capable of eliminating hepatic stages of the parasite, including *Plasmodium vivax* hypnozoites, is becoming essential [9,10].

This work began by an *in vitro* screening of numerous molecules belonging to our chemical library (part of the CNRS French National Chemical Library), toward *P. falciparum*. This library contained numerous human kinase inhibitor-candidates which had previously been synthesized for a research program centered on the design of new anti-cancer agents [11–13]. Among tested compounds, a thieno[3,2-*d*]pyrimidin-4(3*H*)-one derivative appeared as a hit-compound (IC_{50} < 10 μM against the K1 strain). A closely related scaffold had already been proposed by GlaxoSmithKline as a potential antimalarial structure [7] (Fig. 1). In this context, we investigated the antiplasmodial properties of a series of 120 new derivatives [14]. The very recent work reported by Gonzalez Cabrera et al. also demonstrated the great potential of the 2-aminated-6-arylthienopyrimidine scaffold (Fig. 1) in the research for new antimalarial products [15].

2. Results and discussion

Original thieno[3,2-*d*]pyrimidin-4(3*H*)-one derivatives were synthesized by a one pot procedure including a condensation and a cyclization of methyl-3-amino-5-*p*-tolylthiophene-2-carboxylate with ethoxycarbonyle isothiocyanate in DMF [14]. During this reaction, an intermediary thiourea carbamate was formed. This species then reacted with an alkylamine, added to the crude mixture with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, HCl) [16], and triethylamine [17]. As an example, using terbutylamine, this process led to the formation of 2-(*tert*-butylamino)-6-*p*-tolylthieno[3,2-*d*]pyrimidin-4(3*H*)-one **1** in 70% yield (Scheme 1).

Compound **1** was initially evaluated *in vitro* for its antiplasmodial profile against a chloroquine sensitive (3D7) and a multi-drug resistant (K1) *P. falciparum* strains. The evaluation of *in vitro* cytotoxicity was performed on two complementary adherent cancer cell lines: HepG2 and CHO. HepG2 is a commonly used human-derived hepatocarcinoma cell line expressing many of the hepatocyte-specific metabolic enzymes. The aim of this assay using HepG2 in addition to CHO cells was to evaluate the impact of metabolic activation of the tested compounds on cell viability [18] (Table 1).

Based on these experimental results, compound **1** was used for SAR studies and three regions were probed within this chemical structure: the amine region A, the substitution of the phenyl moiety B and the nature of the cycle C (Fig. 2). All derivatives described below were prepared by using the general route presented for the preparation of compound **1** in Scheme 1.

From the structure of hit-compound **1**, primary, secondary and tertiary amine substituents were explored in region A (Table 2). Compounds including a secondary amine at position 2 were selected as the best derivatives since others (compounds **2**, **14**–**18**) showed poor solubility in the cell culture medium, a high toxicity toward the HepG2 cells and/or no antiplasmodial activity. However, although the compounds including a secondary amine at position 2 (compounds **3**, **8**–**13**) did not appear to be cytotoxic, the substitution of the nitrogen atom of the amine group by a longer aliphatic chain notably increased the cytotoxicity (compounds **4**–**7**). Among all tested substituents, *tert*-butylamine (compound **1**) and isopropylamine (compound **8**) provided the best antiplasmodial profiles.

A SAR study was also conducted for region B (Table 3). The suppression of the methyl substituent of the phenyl ring led to a reduction of the potency (compound **27**). The substitution of the same methyl group by an electron-withdrawing group at the *para* position, such as chlorine (compounds **20**, **24**), bromine (compounds **19**, **23**), fluorine (compounds **21**, **25**), or by an electron-donating group, such as methoxy (compounds **22**, **26**), were tolerated mainly with isopropylamine at region A. However, a significant loss in potency was observed if the phenyl group was suppressed (compounds **28**, **29**) or moved at position 7 of the thieno[3,2-*d*]pyrimidin-4(3*H*)-one ring (compounds **32**–**38**). Finally, two analogs derived from compound **1**, 2-(*tert*-butylamino)-6-*m*-tolylthieno[3,2-*d*]pyrimidin-4(3*H*)-one **30** and 2-(*tert*-butylamino)-6-*o*-tolylthieno[3,2-*d*]pyrimidin-4(3*H*)-one **31** were 3–7 fold less potent than the hit-compound and also more cytotoxic.

We finally explored the SAR based on region C by modifying the heterocyclic core (Table 4). As various substituted quinazolines have already been described as antiplasmodial agents [19–23], we particularly studied the replacement of the thiophene ring by a benzene one, resulting in quinazoline analogs (Table 4). This modification led to a significant loss of activity and widely

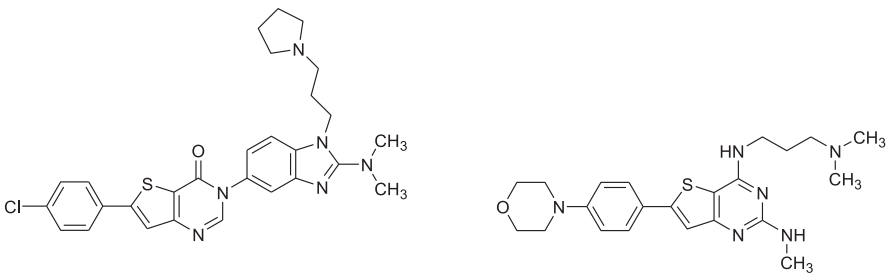
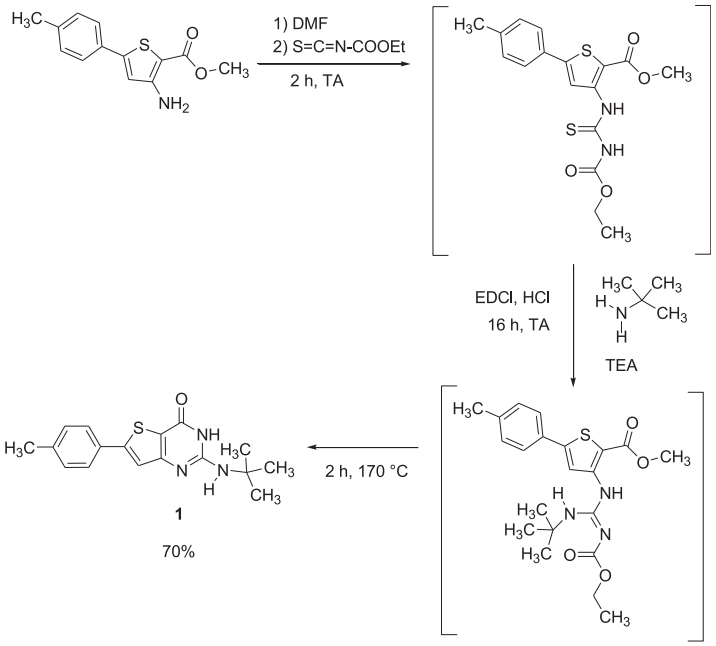


Fig. 1. Thieno[3,2-*d*]pyrimidine derivatives described as antiplasmodial compound by GlaxoSmithKline [7] and Gonzalez Cabrera et al. [15].



Scheme 1. Synthesis of 2-(*tert*-butylamino)-6-*p*-tolylthieno[3,2-*d*]pyrimidin-4(3*H*)-one **1**.

Table 1
Antiplasmodial profile of compound **1**.

Drug	Cytotoxicity[a] (μM)		Antiplasmodial activity[a] (μM)		3D7 strain SI[b]	K1 strain SI[b]
	HepG2 CC_{50}	CHO CC_{50}	3D7 IC_{50}	K1 IC_{50}		
1	25.6 (±3.1)	27.5 (±2.8)	0.14 (±0.01)	0.2 (±0.02)	183	128
Doxorubicin[c]	0.2	0.6	–	–	–	–
Chloroquine[d]	30	155	0.04	0.5	750	60
Doxycycline[d]	20	–	–	5.0	–	4
Atovaquone[d]	>15.6[e]	–	–	0.0013	–	>12,000

[a] The values are mean ± SD of three independent experiments.
[b] Selectivity index (SI) was calculated according to the following formula: $SI = CC_{50}$ (HepG2)/IC_{50}.
[c] Doxorubicin was used as reference drug compound for human cell toxicity.
[d] Chloroquine, doxycycline and atovaquone were used as reference antiplasmodial drug compounds.
[e] Compound could not be tested at a higher concentration because of its lack of solubility in the cell culture medium.

increased the cytotoxicity, indicating the importance of the thieno[3,2-*d*]pyrimidin-4(3*H*)-one core for antiplasmodial profile; variation in the position of the tolyle group on the quinazoline core (compounds **39** and **40**) had no significant influence.

Global conclusion of the SAR studies from compound **1** was that the precise structure of our *in vitro* antiplasmodial pharmacophore corresponded to the 2-amino-6-*p*-substituted phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one, carrying either a *tert*-butylamine substituent at position 2 and a Me or Cl substituent at the para position of the phenyl moiety, or an isopropylamine substituent at position 2 and a Me, MeO, Br, Cl or F substituent at the para position of the phenyl moiety (Fig. 3).

In an attempt to improve the solubility of this series, various salts were prepared and evaluated (Fig. 4). It then appeared that hydrochloride or fumarate salts were favorable for the antiplasmodial activity, the corresponding salts displaying K1 IC_{50} values ranging from 45 nM (**1a**) to 800 nM (**8a** and **20a**), compared with chloroquine (K1 IC_{50} = 500 nM) and atovaquone (K1 IC_{50} = 1.3 nM) used as reference drugs [14] (Fig. 4).

Exploring the antiparasitic spectrum of activity of this molecular scaffold toward other protozoa, compounds **1**, **8** and **20** were then evaluated *in vitro* against *Toxoplasma gondii* and the promastigote stage of *Leishmania donovani*. No activity was revealed against these parasites (IC_{50} > 10 μM) in comparison with reference drugs (Table 4). Therefore, contrary to doxycycline [24] or atovaquone [25,26], this series exhibits a selective antiplasmodial profile.

Then, to further investigate the toxicological profile of this promising series, compounds **1**, **8** and **20** were evaluated for their mutagenicity *via* the Ames test [27,28], at a concentration of 2.5 mM on two distinct *Salmonella typhimurium* strains (TA97A and TA100), in two different conditions, with and without metabolic activation (S9 mix). These compounds were also evaluated for their genotoxicity by the DNA-methyl green test [29]. These molecules were neither mutagenic nor genotoxic (Table 5).

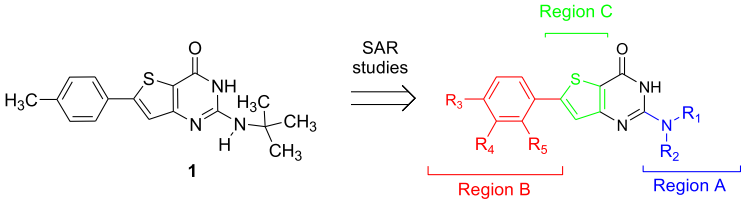
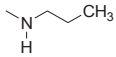
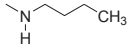
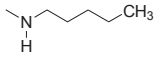
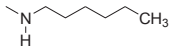
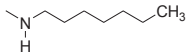
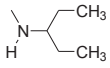
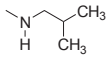
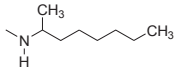
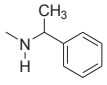
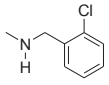
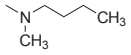
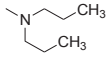
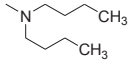
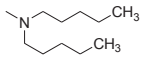


Fig. 2. SARs from compound **1**.

Table 2
SARs of region A.

Compound	NR_1R_2	Cytotoxicity[a] HepG2 CC_{50} (μM)	Antiplasmodial activity[a] K1 IC_{50} (μM)	SI[b]
1		25.6 (±3.1)	0.2 (±0.02)	128
2		–[e]	–[e]	–
3		>62.5[f]	>5[f]	–
4		6.0 (±1.1)	>5[f]	–
5		4.2 (±1.8)	>5[f]	–
6		10.6 (±3.9)	>5[f]	–
7		6.8 (±1.4)	>5[f]	–
8		49.4 (±1.0)	0.8 (±0.5)	62
9		101.3 (±10.1)	>5[f]	–
10		21.6 (±6.1)	>5[f]	–
11		19.4 (±0.3)	>5[f]	–
12		9.2 (±2.8)	>5[f]	–
13		32.6 (±3.8)	>5[f]	–
14		3.0 (±1.1)	>5[f]	–
15		–[e]	–[e]	–
16		–[e]	–[e]	–
17		–[e]	–[e]	–
18		–[e]	–[e]	–
Doxorubicin[c]		0.2	–	–
Chloroquine[d]		30	0.5	60
Doxycycline[d]		20	5	4
Atovaquone[d]		>15.6[f]	0.0013	>12,000

[a] The values are means ± SD of three independent experiments.
[b] Selectivity index (SI) was calculated according to the following formula: $SI = CC_{50}/IC_{50}$.
[c] Doxorubicin was used as reference drug compound for human cell toxicity.
[d] Chloroquine, doxycycline and atovaquone were used as reference antiplasmodial drug compounds.
[e] Compound could not be tested because of its lack of solubility in the cell culture medium.
[f] No activity was observed at the highest concentration tested.

The most active antiplasmodial compound **1a** in the series was also tested *in vivo* against a chloroquine sensitive strain of *Plasmodium berghei* (NK65). This preliminary evaluation (one concentration of compound **1a**, one administration route, groups of 5 animals) of the antimalarial potential was determined by the classical Peters four-day curative standard test [30–32]. Thus, 3 groups of five infected mice were compared: untreated (control group) and treated by intra peritoneal route with 2.5 mg/kg of either compound **1a** or chloroquine (reference drug, Sigma), twice a day, for four consecutive days beginning on the day of infection. On day 4, Giemsa-stained thin blood smears were made for each mouse, and parasitemia was estimated by visual numeration. These preliminary results are encouraging, compound **1a** being responsible for a 45% reduction in parasitemia in comparison with the chloroquine control at the same dose: 85% (Table 6).

The effect of compound **1a** on the erythrocytic life cycle of the K1 *P. falciparum* strain was also evaluated from synchronized parasite cultures (Fig. 5) [33]. During the erythrocytic cycle, the highest activity of **1a** was noted between 0 and 8 h for the highest concentration ($5 \times IC_{50}$), with 41% reduction in parasitemia compared to negative control. This result suggests that compound **1a** is active against ring stage parasites, and may inhibit the invasion of new erythrocytes. We noticed that this activity remained until the 32nd hour of the erythrocytic life cycle with 30% reduction in parasitemia for the highest tested concentration. This period of activity corresponds to the ring and trophozoïte forms of the parasites. All these observations suggest that compound **1a** does not act on a single target during the erythrocytic life cycle, and may simultaneously employ several mechanisms of action.

In the development of new antimalarial compounds, the assessment of the effect toward the hepatic stage of the parasite remains a key point, since new agents that act at stages of the parasite life cycle in addition to the erythrocytic stage are sorely needed. To investigate the efficacy of our molecules toward the liver stage of *Plasmodium*, we performed an *in vitro* assay in which hepatic HepG2-CD81 cells are infected by *Plasmodium yoelii* 17X NL sporozoites [34] (Table 7). In this assay, atovaquone and primaquine (as a member of the 8-aminoquinoline family), two of the very few molecules showing good hepatic activity, were used as reference-drugs. Indeed, atovaquone and primaquine are known to be active against the blood and liver stages of malaria [35], even if primaquine also has described stage activity against mature gametocytes of *P. falciparum* [36]. Very interestingly, lead-compounds **1a**, **8a** and **20a** (more especially **1a**) displayed IC_{50} values (35–120 nM) close to the excellent level of atovaquone (9 nM) and much better than that of primaquine (1160 nM).

Furthermore, an investigation was carried out on the lead-compounds in an attempt to elucidate their mechanism(s) of action. The mechanisms studied were those known for the most important commercial antimalarial drugs: chloroquine [38], atovaquone [39], pyrimethamine [40] and artesunate [41]. Inhibition of hemozoin polymerization is an important target for 4-aminoquinoline-based antimalarials such as chloroquine and amodiaquine, two well-known blood schizonticidals. Using the microtitre-base assay [38], out of the tested compounds, only **1** showed some β-hematin inhibition activity (hemozoin polymerization IC_{50} = 21.6 mM), compared with chloroquine (hemozoin polymerization IC_{50} = 1.46 mM) used as a reference drug (Table 8).

The antimalarial mechanism of action of atovaquone, an active drug against blood and liver stages that targets *Plasmodium* respiration, consists in the depolarization of the mitochondrial membrane. By using a very low concentration (2 nM) of the cationic, lipophilic and fluorescent probe, 3,3′-dihexyloxacarbocyanide iodide ($DiOC_6$), a measurement of the mitochondrial membrane potential in infected erythrocytes by flow cytometry is defined as an

Table 3
SAR of region B.

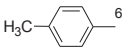
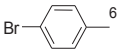
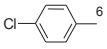
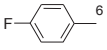
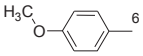
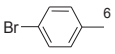
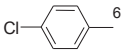
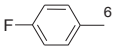
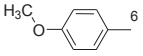
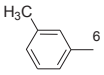
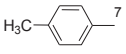
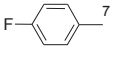
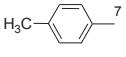
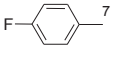
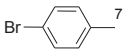
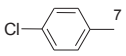
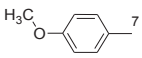
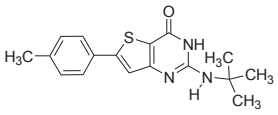
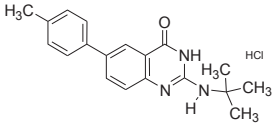
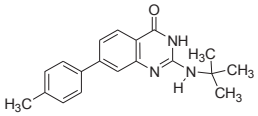
Compound	Region A	Region B	Cytotoxicity[a] HepG2 CC_{50} (μM)	Antiplasmodial activity[a] K1 IC_{50} (μM)	SI[b]
1			25.6 (±3.1)	0.2 (±0.02)	128
19			1.4 (±0.6)	>5[f]	–
20			15.0 (±2.7)	0.8	19
21			5.1 (±1.0)	>5[f]	–
22			32.3 (±2.2)	>5[f]	–
23			13.4 (±1.9)	1.7	9
24			>15.6	0.6	>26
25			>25[f]	0.5	>66
26			27.1 (±3.9)	0.5	136
27			12.5 (±2.5)	1.0	10
28			8.4 (±3.5)	>5[f]	–
29			23.9 (±2.1)	>5[f]	–
30			14.1 (±1.4)	3.6	3
31			4.0 (±1.1)	1.7	2
32			1.4 (±0.4)	>5[f]	–
33			2.3 (±0.2)	>5[f]	–
34			8.8 (±0.8)	2.7	3
35			>15.6[f]	>5[f]	–

Table 3 (*continued*)

Compound	Region A	Region B	Cytotoxicity[a] HepG2 CC_{50} (μM)	Antiplasmodial activity[a] K1 IC_{50} (μM)	SI[b]
36			6.5 (±0.1)	>5[f]	–
37			7.1 (±0.6)	>5[f]	–
38			27.8 (±4.1)	>5[f]	–
Doxorubicin[c]			0.2	–	–
Chloroquine[d]			30	0.5	60
Doxycycline[d]			20	5	4
Atovaquone[d]			>15.6[e]	0.0013	>12,000

[a] The values are means ± SD of three independent experiments.
[b] Selectivity index (SI) was calculated according to the following formula: $SI = CC_{50}/IC_{50}$.
[c] Doxorubicin was used as reference drug compound for human cell toxicity.
[d] Chloroquine, doxycycline and atovaquone were used as reference antiplasmodial drug compounds.
[e] Compound could not be tested because of its lack of solubility in the cell culture medium.
[f] No activity was observed at the highest concentration tested.

Table 4
SAR of region C.

Drug	Chemical structure	Cytotoxicity[a] HepG2 CC_{50} (μM)	Antiplasmodial activity[a] K1 IC_{50} (μM)	SI[b]
1		25.6 (±3.1)	0.2 (±0.02)	128
39		0.49 (±0.9)	>5[f]	<10.2
40		0.98 (±0.6)	>5[f]	<5.1
Doxorubicin[c]		0.2	–	–
Chloroquine[d]		30	0.5	60
Doxycycline[d]		20	5	4
Atovaquone[d]		>15.6[e]	0.0013	>12,000

[a] The values are means ± SD of three independent experiments.
[b] Selectivity index (SI) was calculated according to the following formula: $SI = CC_{50}/IC_{50}$.
[c] Doxorubicin was used as reference drug compound for human cell toxicity.
[d] Chloroquine, doxycycline and atovaquone were used as reference antiplasmodial drug compounds.
[e] Compound could not be tested because of its lack of solubility in the cell culture medium.
[f] No activity was observed at the highest concentration tested.

alternative *in vitro* assay to study the mitochondrial function in malarial parasites [39]. Atovaquone, used as reference drug, caused a depolarization of the plasmodial mitochondria membrane, as seen in the flow cytometric profile. At K1 IC_{50}, none of the tested compounds (**1**, **8**, **20**) had any effect on malarial mitochondrial membrane potential (Fig. 6).

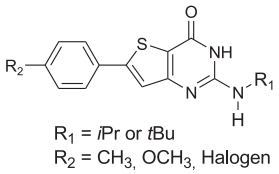


Fig. 3. Antiplasmodial pharmacophore in 6-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one series.

We also evaluated *in vitro* the effect of lead-compounds **1**, **8** and **20** toward their *P. falciparum* dihydrofolate reductase (*Pf*DHFR) inhibitory activity by using two antifolinic erythrocytic schizonticidals, pyrimethamine and cycloguanil, as reference drugs. None of tested compounds revealed any inhibition of this parasitic target by using both the spoke assay and the 96-well microtiter plates assay [40] (Table 9).

Finally, we tested the ability of these compounds to generate free radicals, using artesunate as reference, an antimalarial drug that targets pathogenic blood stage parasites and immature gametocytes [36], proposed to utilize that molecular mechanism of action. The general procedure for this assay was similar to the

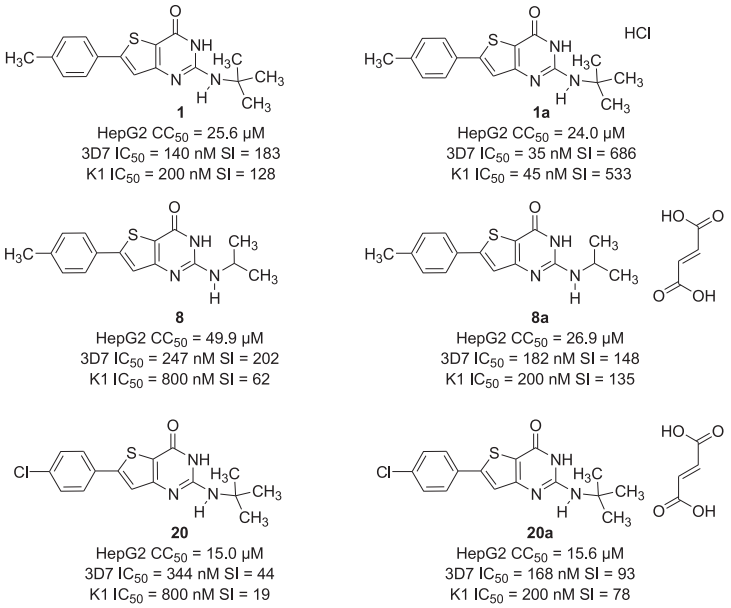


Fig. 4. Promising compounds **1**, **8** and **20** and their salts **1a**, **8a** and **20a**.

Table 5
Complementary biological results displayed by lead-compounds **1**, **8** and **20**.

Drug	Antileishmanial activity promastigotes IC_{50} (μM)	Antitoxoplasmic activity IC_{50} (μM)	Mutagenicity at 2.5 mM (Ames test)		Genotoxicity (DNA-methyl green test) DC_{50}[g] (mM)
			Without S9 mix[f]	With S9 mix[f]	
1	>10[h]	>10[h]	Negative	Negative	>25[i]
8	>10[h]	>10[h]	Negative	Negative	>25[i]
20	>10[h]	>10[h]	Negative	Negative	>25[i]
Amphotericine B[a]	0.08	–	–	–	–
Pyrimethamine[b]	–	2	–	–	–
ICR170[c]	–	–	Positive	–	–
NaN_3[c]	–	–	Positive	–	–
Benzo[*a*]pyrene[d]	–	–	–	Positive	–
Daunorubicine[e]	–	–	–	–	0.001

[a] Amphotericine B was used as reference antileishmanial drug compound.
[b] Pyrimethamine was used as reference antitoxoplasmic drug compound.
[c] ICR170 (TA97A *S. typhimurium* strain) and NaN_3 (TA100 *S. typhimurium* strain) were used as reference mutagenic drug compounds without metabolic activation.
[d] Benzo[*a*]pyrene (TA97A and TA100 *S. typhimurium* strains) was used as reference mutagenic drug compound with metabolic activation.
[e] Daunorubicine was used as reference genotoxic drug compound.
[f] S9 mix was used to test mutagenicity after metabolic activation.
[g] DC_{50} refers to amount of compound required to displace 50% of the methyl green from DNA-methyl green complex.
[h] No activity was observed at the highest concentration tested.
[i] No toxicity was observed at the highest concentration tested.

in vitro determination of the antiplasmodial activity with addition of a radical scavenger, ascorbic acid, in the parasite culture medium. None of the tested compounds seemed to produce free radicals, their respective K1 IC_{50} being similar in the presence or in absence of ascorbic acid (Table 10), compared with artesunate.

In summary of all these mechanistic experiments, we conclude that the tested compounds do not use the already described mechanisms of action of commercialized antimalarial drugs, which suggests that they might be appropriate partner drugs in a combination. Further work will be necessary to identify the mechanism(s) of action of these newly identified antimalarial thienopyrimidines. Among possible targets which have to be seriously considered, regarding the thienopyrimidine core of these molecules, we will focus on the *P. falciparum* dihydroorotate dehydrogenase (*Pf*DHOD) [42] and also on several *P. falciparum* kinases which have already been reported as relevant targets [7,43–46] remembering that these novel compounds were initially identified by screening a chemical library containing molecules which had been designed as human kinase inhibitors.

Table 6
Parasitemia of *Plasmodium berghei* infected mice not treated and intraperitoneally treated with 5 mg/kg of chloroquine or compound **1a** in a classic four-day suppressive test.

	Untreated mice	Chloroquine[a]	**1a**
		2 × 2.5 mg/kg	2 × 2.5 mg/kg
Parasitemia	9%	1.5%	4.9%

[a] Chloroquine was used as reference antimalarial drug compound.

3. Conclusion

The present work globally demonstrated that 2-aminated-6-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one derivatives present a real promising selective antimalarial profile, targeting both the

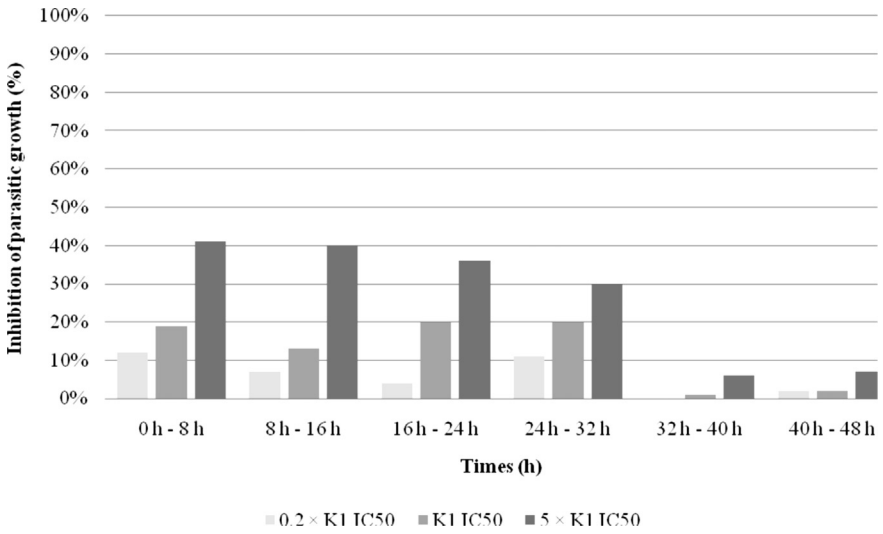


Fig. 5. Effect of compound **1a** on the erythrocytic life cycle of *P. falciparum* K1 strain.

erythrocytic and hepatic stages of the parasite through an original mechanism of action which remains to be elucidated. Complementary investigations including more complete *in vivo* and mechanistic studies, will then be carried out in a view to address the pharmaceutical development of such novel antimalarial scaffold.

4. Experimental

4.1. Chemistry

4.1.1. General material and methods

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a Kofler melting point apparatus.^{1}H and ^{13}C NMR spectra were recorded on a JEOL Lambda 400 spectrometer and BRUKER AVANCE III 500 MHz with chemical shifts expressed in parts per million (in DMSO-d_6) downfield from tetramethylsilane as an internal standard and coupling in Hertz. Elemental analyses were performed at the "Institut de Recherche en Chimie Organique Fine" (Rouen, France).

4.1.2. General procedure for the synthesis of 2-alkyl (dialkyl)aryl-2-aminothieno[3,2-d]pyrimidin-4(3H)-one derivatives (**1**–**40**)

To a solution of methyl-3-amino-5-*p*-tolylthiophene-2-carboxylate in DMF (40 mL) was added ethoxycarbonyle isothiocyanate (1 eq). The solution was left stirring at room temperature overnight. During this reaction, a thiourea carbamate intermediate was formed and was not isolated. To the reaction mixture were then added triethylamine (3 eq), terbutylamine (2 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDCI, HCl) (1.2 eq). The solution was then heated under reflux for 2 h. The solvent was removed under reduced pressure. Water (10 mL) was then added to the reaction mixture and the precipitated solid obtained was filtered, washed with diethyl ether and recrystallized from acetonitrile to give the corresponding 2-alkyl(dialkyl)aryl-2-aminothieno[3,2-*d*]pyrimidin-4(3*H*)-one derivative.

4.1.3. General procedure for the synthesis of 2-tert-butylamino-6-p-tolyl-3H-quinazolin-4-one, hydrochloride (**39**)

To a solution of methyl 2-amino-5-(4-methylphenyl)benzoate (1 g, 4.14 mmol) in DMF (8 mL) were added ethoxycarbonyl isothiocyanate (0.49 mL, 1 eq). The solution was left stirring at room temperature overnight. During this reaction, a thiourea carbamate intermediate was formed and was not isolated. To the reaction mixture was then added triethylamine (1.73 mL, 3 eq), terbutylamine (0.87 mL, 2 eq), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDCI, HCl) (952 mg, 1.2 eq). The solution was then heated under reflux for 2 h. The solvent was removed under reduced pressure. Water (5 mL) was then added to the reaction mixture and the precipitate obtained was filtered, washed with diethyl ether and recrystallized from acetonitrile. The residue obtained (600 mg) was dissolved into ethanol (35 mL) and hydrochloric acid (0.2 mL, 1.2 eq) was added. The reaction was then heated under reflux for 1 h. The precipitate obtained was filtered and recrystallized from acetonitrile to give **39**, as a white solid (320 mg, 48%).

Table 7
Liver stage activity displayed by the most promising compounds **1a**, **8a** and **20a**.

Drug	*P. yoelii* IC_{50} (nM)	HepG2-CD81 CC_{50} (μM)	Selective index[a]
1a	35	>143[c]	>4086
8a	120	>111[c]	>925
20a	60	>120[c]	>2000
Atovaquone[b]	9[d]	9.5[d]	1056[d]
Primaquine[b]	1160[d]	6,1[d]	5.3[d]
Chloroquine	1650[d]	1.6[d]	0.97[d]
Artemisinin	>10,000[d]	>10[d]	–

[a] Therapeutic index was calculated according to the following formula: HepG2-CD81 CC_{50}/*P. yoelii* IC_{50}.
[b] Atovaquone and primaquine were used as reference drug compounds active during the *Plasmodium* liver stage.
[c] No toxicity was observed at the highest concentration tested.
[d] Data already published [37].

Table 8
Inhibition of hemozoin polymerization *in vitro* displayed by compounds **1**, **8** and **20**.

Drug	Hemozoin polymerization IC_{50} (mM)
1	21.6
8	>25[b]
20	>25[b]
Chloroquine[a]	1.46

[a] Chloroquine was used as reference drug inhibiting hemozoin polymerization.
[b] No inhibition was observed at the highest concentration tested.

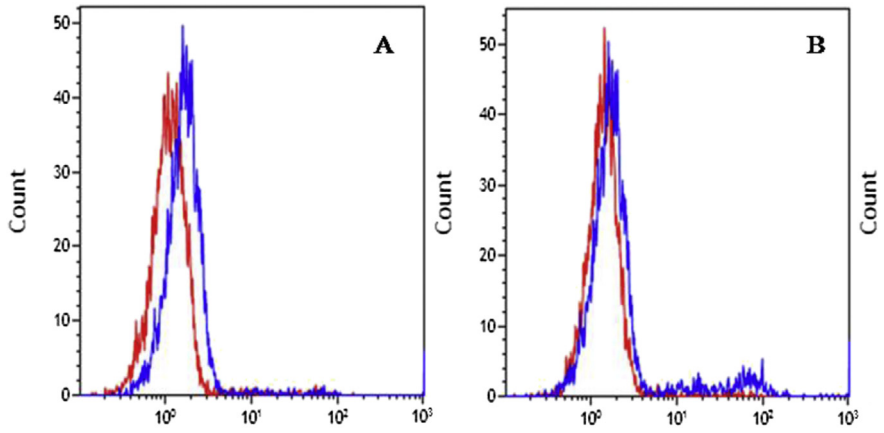


Fig. 6. Cytometric profiles in the depolarization of the mitochondrial membrane (red curve: without compound; blue curve: **A** = Atovaquone used as reference drug compound depolarizing mitochondrial membrane or **B** = Compound **1** at K1 IC_{50}). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 9
P. falciparum dihydrofolate reductase (*Pf*DHFR) inhibitory activity *in vitro* assay.

Compound	*P. falciparum* DHFR inhibitory activity IC_{50} (μM)
1	>500[b]
8	>250[b]
20	>250[b]
Pyrimethamine[a]	2.8
Cycloguanil[a]	5

[a] Pyrimethamine and cycloguanil were used as reference drugs inhibiting *Pf*DHFR.
[b] No inhibitory activity was observed at the highest tested concentration.

*4.1.4. General procedure for the synthesis of 2-tert-butylamino-6-(p-tolyl)-3H-thieno[3,2-d]pyrimidin-4-one, hydrochloride (**1a**)*

To a solution of **1** (2.5 g, 7.97 mmol) in ethanol (150 mL) was added hydrochloric acid (0.8 mL, 1.2 eq). The reaction was then heated under reflux for 1 h. The precipitate obtained was filtered and recrystallized from acetonitrile to give **1a** as a yellow solid (2.4 g, 85%).

*4.1.5. General procedure for the synthesis of 2-tert-butylamino-6-(p-chlorophenyl)-3H-thieno[3,2-d]pyrimidin-4-one, fumarate (**20a**)*

To a solution of **20** (500 mg, 1.49 mmol) in isopropanol (30 mL) was added fumaric acid (208 mg, 1.2 eq). The reaction mixture was then heated under reflux for 3 h. The precipitate obtained was filtered and recrystallized from acetonitrile to give **20a** as a white solid (500 mg, 74%).

*4.1.6. General procedure for the synthesis of 2-isopropylamino-6-(p-tolyl)-3H-thieno[3,2-d]pyrimidin-4-one, fumarate (**8a**)*

To a solution of **8** (400 mg, 1.33 mmol) in isopropanol (25 mL) was added fumaric acid (186 mg, 1.2 eq). The reaction mixture was then heated under reflux for 3 h. The precipitate obtained was filtered and recrystallized from acetonitrile to give **8a** as a white solid (430 mg, 77%).

Table 10
Free radical production assay.

Compound	Antiplasmodial activity K1 IC_{50} (μM)	Antiplasmodial activity in presence of ascorbic acid (800 μM)
		Ascorbic acid K1 IC_{50} (μM)
1	0.2	0.2
8	0.8	0.9
20	0.8	0.8
Artesunate[a]	<0.1	0.7

[a] Artesunate was used as reference drug compound generating radicals.

4.2. Biological evaluation

4.2.1. In vitro cytotoxicity evaluation on CHO and HepG2 cell lines

CHO and HepG2 cell lines were maintained at 37 °C, 6% CO_2, 14% O_2, 80% N_2, with 90% humidity in RPMI supplemented with 10% fœtal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (complete RPMI medium). *In vitro* cytotoxicity evaluation on CHO and HepG2 cell lines was performed according to the method described by Mosman [47] with slight modifications. Briefly, 5.10^3 cells in 100 μL of culture medium (RPMI + 10% CO_2) were inoculated into each well of 96-well plates and incubated at 37 °C in a humidified 6% CO_2, 14% O_2, 80% N_2 atmosphere. After 24 h incubation, 100 μL of medium with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated from 24 h (CHO) to 72 h (HepG2). Duplicate assays were performed for each sample. At the end of the treatment and incubation, each plate-well was microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. Then, 10 μL yellow MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) solution (5 mg MTT/mL in PBS) was added to each well with 100 μL of medium without fœtal bovine serum. Cells were incubated for 2 h at 37 °C to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After 2 h, the MTT solution was aspirated and DMSO (100 μL) was added to each well to dissolve the resulting blue formazan crystals. Plates were then shaken vigorously (300 rpm) for a few minutes. The absorbance was measured at 570 nm with 630 nm as reference wavelength, using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and doxorubicin as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentrations (CHO CC_{50} and HepG2 CC_{50}) were determined by non-linear regression analysis processed on dose–response curves, using the Table Curve software 2D v.5.0. CC_{50} values represent the mean value calculated from three independent experiments.

4.2.2. Antiplasmodial activity in vitro assay [48,49]

The evaluation of compounds' antiplasmodial activity was conducted *in vitro* on one to three culture-adapted *P. falciparum* strains (3D7 sensible; K1 resistant to chloroquine, pyrimethamine

and proguanil). *P. falciparum* strains were maintained in fresh A+ human erythrocytes at 2.5% haematocrit in complete medium (RPMI 1640 with 25 mM HEPES, 25 mM $NaHCO_3$, 10% of A+ human serum) at 37 °C under reduced O_2 atmosphere (gas mixture 6% CO_2, 14% O_2, 80% N_2). Parasitaemia was maintained daily between 1% and 6%. The *P. falciparum* drug susceptibility test was carried out by comparing quantities of DNA in treated and control cultures of parasite in human erythrocytes according to an SYBR Green I fluorescence-based method using a 96-well fluorescence plate reader. Parasite culture was synchronized at ring stage with 5% sorbitol. Compounds dissolved in DMSO (final concentration less than 0.5% v/v) were incubated in a total assay volume of 200 μL (RPMI, 2% haematocrit and 1% parasitaemia) for 72 h in a humidified atmosphere (6% CO_2, 14% O_2, 80% N_2) at 37 °C, in 96-well flat-bottom plates. Duplicate assays were performed for each sample. After incubation, 125 μL supernatant was discarded and cells were washed twice with 150 μL 1X PBS. 15 μL re-suspended cells were transferred to 96-well flat bottom non-sterile black plates (Greiner Bio-one). 15 μL of the SYBR Green lysis buffer (2XSYBR Green, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100) was added to each well. Negative control (treated by DMSO) and positive controls (doxycycline and chloroquine) were added to each set of experiments. Plates were incubated for 0.25 h at 37 °C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 497 and 520 nm respectively. The concentrations of compounds required to induce a 50% decrease in parasite growth (IC_{50}) were calculated from three independent experiments. The selectivity indexes presented correspond to the ratios between, respectively, the cytotoxicity and the antiplasmodial activity. They are calculated as follows: SI = CC_{50} (HepG2)/IC_{50} (3D7 or K1).

4.2.3. Antileishmanial activity in vitro assay

L. donovani strain was maintained at 27 °C, 6% CO_2, 14% O_2, 80% N_2, with 90% humidity in Schneider's medium supplemented with 10% fœtal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (complete Schneider's medium). The *L. donovani* promastigotes drug susceptibility test was carried out by using the method described by Mosman [47] with slight modifications. Briefly, 1.10^6 cells in log-phase in 100 μL of culture medium (complete Schneider's medium) were inoculated into each well of 96-well plates. 100 μL of cells in culture medium with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated at 27 °C in a humidified 6% CO_2, 14% O_2, 80% N_2 atmosphere for 72 h. Amphotericin B and pentamidine were used as reference drugs. Duplicate assays were performed for each sample. At the end of the treatment and incubation, the medium was aspirated from wells and 10 μL yellow MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) solution (5 mg MTT/mL in PBS) was added to each well with 100 μL of medium without fœtal bovine serum. Cells were incubated for 6 h at 27 °C to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After 6 h, the MTT solution was aspirated and DMSO (100 μL) was added to each well to dissolve the resulting blue formazan crystals. Plates were then shaken vigorously (500 rpm) for a few minutes. The absorbance was measured at 570 nm with 630 nm as reference wavelength, using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and reference drugs as positive controls. The *L. donovani* promastigotes drug susceptibility was calculated as percentage of control (cells incubated without compound). The 50% inhibitory concentration (*Ld* IC_{50}) represents the concentration of compound required to induce a 50% decrease of parasite growth. It was determined by non-linear regression analysis processed on dose–response curve, using the Table Curve software 2D v.5.0. *Ld* IC_{50} values represent the mean value calculated from three independent experiments.

4.2.4. Antitoxoplasmic activity in vitro assay

The effects of the tested compounds on the growth of *T. gondii* tachyzoites (PRU-b-Gal strain, kindly provided by Pr. I. Villena, Reims, France) were assessed by a colorimetric microtiter assay according to the method of McFadden et al. [50]. Briefly, tachyzoites were maintained by serial passage in confluent human foreskin fibroblast (HFF) monolayer (a gift of Pr. I. Dimier-Poisson, Tours, France). For assay, 96-well microtiter plates were seeded with 1.10^4 HFF cells and allowed to grow to confluence in complete RPMI medium at 37 °C with 6% CO_2, 14% O_2, 80% N_2 and 90% humidity. Cell monolayers were infected with 2.10^3 parasites per well and incubated at 37 °C in a humidified 6% CO_2, 14% O_2, 80% N_2 atmosphere for 3 h. Then, various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v) were incorporated in triplicate. Pyrimethamine was used as the reference drug compound. Appropriate controls treated by DMSO or pyrimethamine were added to each set of experiments. Negative control consisted in cell monolayers incubated without parasite and drug. After a 96-h incubation period at 37 °C in a humidified 6% CO_2, 14% O_2, 80% N_2 atmosphere, 20 μL of chlorophenol red-β-D-galactopyranoside (CPRG) were added to each well to give a final concentration of 500 μM. The plates were incubated at 37 °C for an additional 24 h. Then, the absorbance was measured at 570 nm with 630 nm as reference wavelength, using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and reference drugs as positive controls. The *T. gondii* drug susceptibility was calculated as percentage of control (cells incubated without compound). The 50% inhibitory concentration (*Tg* IC_{50}) represents the concentration of compound required to induce a 50% decrease of parasite growth. It was determined by non-linear regression analysis processed on dose–response curve, using the Table Curve software 2D v.5.0. *Tg* IC_{50} values represent the mean value calculated from three independent experiments.

4.2.5. Ames test

Mutagenicity test was carried out by using a modified version of the liquid incubation assay of the classical Ames test [27,28]. *S. typhimurium* tester strains (TA97a and TA100) were grown overnight in an Oxoid nutrient broth. After this period, 2.5–25 mM DMSO solutions of the tested drugs were added to 0.1 mL of culture and incubated with and without 4% S9 mix for 1 h at 37 °C with shaking. After this period, 2 mL of molten top agar were added and the mixture was transferred onto Vogel-Bonner agar plates. After 48 h at 37 °C in the dark, the number of spontaneous and drug induced revertants per plate was determined for each dose with a laser bacterial colony counter. A product is considered mutagenic when it induces a twofold increase of the number of revertants compared with the spontaneous frequency.

4.2.6. Microtiter DNA-methyl green assay

Microtiter DNA-methyl green assay was carried out by using the method described by Burres [29]. Before use in an assay, DNA-methyl green solution was prepared as follows: 4 mg of DNA-methyl green was first dissolved in a 20 mL Tris–HCl buffer 0.05 M, pH 7.5 with 7.5 mmol/L of magnesium sulfate. This DNA-solution was then shaken at 37 °C for 24 h. 2 μL various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v) was incorporated into the wells of 96-well microtiter plate. Daunorubicin was used as the reference drug compound. 200 μL DNA-methyl green solution was added to each well and plate was shaken vigorously (300 rpm) for a few minutes. Absorbance was determined at 630 nm, using a BIO-TEK ELx808

Absorbance Microplate Reader. DMSO was used as blank, DNA-methyl green as negative control and daunorubicin as positive one. Plate was incubated at room temperature in darkness for 24 h before a new absorbance measurement. Agents that displace methyl green from DNA are detected spectrophotometrically by a decrease in absorbance at 630 nm. The 50% displacement concentration (DC_{50}) represents the concentration of compound required to induce a 50% displacement of methyl green from DNA.

4.2.7. *In vivo preliminary study*

The *in vivo* antimalarial activity was determined by the classical four-day suppressive test [30] against *P. berghei* NK65. Swiss male mice (Janvier, France), of a mean body weight 20 ± 2 g, were infected with 10^7 parasitized cells in 0.9% saline, on day 0.

Groups of 5 mice were treated by intra peritoneal route from day 0 to day 3 with 2.5 mg/kg twice a day of tested compound. Chloroquine was used as control at 2.5 mg/kg twice a day. The suppressive effects was estimated on day 4, examining Giemsa-stained thin blood smears made from the tail of the treated mice and compared with a control group of mice treated with saline. The stained thin blood smears were examined under × 1000 magnification, and the percentage of parasitized red blood cells was counted on at least 9000 red blood cells observed for each concentrations. Percent growth inhibition of the parasite was calculated by the following formula:

$$[(\text{parasitaemia in control} - \text{parasitaemia with drug}) \times 100]/\text{parasitaemia in control}$$

All experimental animal procedures respect French legislation on laboratory animal use and care (N°2001-464).

4.2.8. *Stage of action in the erythrocytic life cycle*

The K1 *P. falciparum* strain was synchronized to an 8-h period. The method consisted of synchronizing young forms with 5% D-sorbitol [51]. Different concentrations of compound dissolved in DMSO (final concentration less than 0.5% v/v), negative control (treated by DMSO) and positive control (chloroquine) were tested in 24-well plate with culture at 0.8% parasitemia (hematocrit 4%). Cultures were subjected to 8-h pulses (corresponding to 1/6 of the erythrocytic cycle time) with products (0.2 K1 IC_{50}; K1 IC_{50}; 5 K1 IC_{50}). After being pulsed, the cultures were washed three times with culture medium and then returned to normal conditions until the next cycle [33]. At the end of the experiment (the ring stage of the next erythrocytic cycle), parasitemia was determined on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 497 and 520 nm respectively. The results were expressed as an inhibition percentage of parasitic growth.

4.2.9. *Plasmodium liver stages activity assay*

HepG2/CD81 cells [52], were cultured in Dulbecco modified Eagle medium (D-MEM), supplemented with 10% fetal bovine serum heat inactivated, 2 mM L-glutamine, 100 units/mL of penicillin and 100 μg/mL of streptomycin (all from Invitrogen, France). Cells were seeded in 96 well plates coated with 5 μg/mL collagen (rat tail type I, BD Bioscience) at a density of 25.10^3 cells per well. After 24 h of incubation at 37 °C, cells were treated with the various concentrations of molecule **1a**, **8a** or **20a** added in the culture medium and in the same time infected with 5000 sporozoites of *P. yoelii yoelii* (17X NL strain, obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands 14 days after their infective blood meal). Sporozoite-inoculated culture plates were centrifuged for 10 min at 900 × g at 4 °C in order to enhance the infection rate. The treatment was renewed three hours later, after sporozoites penetration into cells, and at 24 h post-infection. At 48 h post-infection cultures were fixed 10 min with cold methanol. Parasite quantification was done by immunofluorescence analysis. Intracellular parasites were stained with a mouse polyclonal serum raised against *Plasmodium* heat shock protein 70 [53], followed by goat anti-mouse Alexa Fluor 488 conjugate (Invitrogen, Molecular Probes) and cell nuclei were stained with 1 μg/mL of diamidino-phenylindole (DAPI; Invitrogen). Fluorescence intensity of DAPI in the different conditions was quantified on a FlexStation (Molecular devices Ltd., UK) at λex = 350 nm and λem = 470 nm. The 50% cytotoxic concentration (CC_{50}) values correspond to the drug concentration at which a 50% reduction of DAPI fluorescence was observed as compared to that observed in the control cultures (control wells consisted on cells treated with the drug solvent final concentration, i.e. 0.5% DMSO). Parasite numbers in the different conditions were counted under a fluorescence microscope with a 200×× magnification. IC_{50} values correspond to the drug concentration at which a 50% reduction in the number of parasites was observed as compared to the number in the control wells.

4.2.10. *Inhibition of heme polymerization in vitro assay [38]*

100 μL of a 4 mM solution of hemozoin, previously dissolved in 0.1 M NaOH, were distributed in 96-well microtiter plates. 50 μL of different concentrations of tested compounds at a drug:heme ratio of between 1:1 to 8:1 were added to triplicate test wells. Hemozoin polymerization was initiated by adding 0.8 mmol of acetic acid (50 μL) at a final pH of 3 and the suspension was incubated at 37 °C for 24 h to allow complete polymerization. Plates were then centrifuged at 3300 g for 15 min and the soluble fraction of unprecipitated material collected (fraction 1). The remaining pellet was resuspended with 200 μL of DMSO to remove unreacted hemozoin. Plates were then centrifuged at 3300 g for 15 min. The DMSO-soluble fraction (fraction 2) was collected and the pellet, consisting of a pure precipitate of β-hemozoin, was dissolved in 0.1 M NaOH (fraction 3) for spectroscopic quantification. A 150 μL aliquot of each fraction was transferred on to a new plate and serial four-fold dilutions in 0.1 M NaOH were performed. The amount of hemozoin was determined by measuring the absorbance at 405 nm using a BIO-TEK ELx808 Absorbance Microplate Reader. A standard curve for hemozoin dissolved in 0.1 M NaOH was used to calculate the amount of porphyrin present in each fraction. The 50% inhibitory concentration (hemozoin polymerization IC_{50}) represents the concentration of compound required to induce a 50% decrease of hemozoin polymerization.

4.2.11. *Depolarization of mitochondrial membrane potential in vitro assay [39]*

Parasitized cells at a concentration of 5.10^6/mL in RPMI 1640 medium containing 1% fœtal bovine serum were incubated with a 2 nM final concentration of DiOC6 for 20 min at 37 °C. At the end of the incubation period the suspension was aliquoted into different tubes of 250 μL each. Different concentrations of tested compounds were added, and the mixture was incubated for an additional 20 min. At the end of the incubation period, each sample was subjected to flow cytometric analysis (Gallios, Beckman Coulter). For each sample 10 000 events were counted at the same flow cytometric setting. Measurements of fluorescence in infected erythrocytes in the presence or in absence of dye were carried out to establish baselines.

4.2.12. *P. falciparum DHFR inhibitory activity in vitro assay*

The drug sensitivity tests used a recombinant *Saccharomyces cerevisiae* culture: the DHFR gene from the budding yeast, *S.*

cerevisiae, has been replaced with the DHFR domain from *P. falciparum* [54]. The drug sensitivity tests were conducted in 96-well microtiter plates by monitoring the growth at an optical density of 660 nm. Each well contained 1.10^4 cells of yeasts of interest in liquid culture in 100 μl of non selective medium (YEPD), and 100 μL of drug solution dissolved in DMSO (final concentration less than 0.5% v/v). The plates were incubated at 30 °C for 48 h. Pyrimethamine was used as reference drug. Duplicate assays were performed for each tested sample. At the end of incubation, plates were shaken vigorously for a few minutes. The absorbance was measured at 660 nm using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and pyrimethamine as positive control. The concentration that inhibited 50% yeast growth (IC_{50}) was determined by non-linear regression analysis processed on dose–response curve.

4.2.13. Production of free radicals in vitro assay

The evaluation of compounds' ability to generate free radicals was conducted *in vitro* on *P. falciparum* K1 strains maintained in standard conditions previously described. The test was carried out by comparing quantities of DNA in treated and control cultures of parasite in human erythrocytes according to an SYBR Green I fluorescence-based method using a 96-well fluorescence plate reader, in presence of ascorbic acid used as radicals trap. Compounds dissolved in DMSO (final concentration less than 0.5% v/v) were incubated in presence of 800 μM ascorbic acid in a total assay volume of 200 μL (RPMI, 2% haematocrit and 1% parasitaemia) for 72 h in a humidified atmosphere (6% CO_2, 14% O_2, 80% N_2) at 37 °C, in 96-well flat-bottom plates. Duplicate assays were performed for each sample. After incubation, 125 μL supernatant was discarded and cells were washed twice with 150 μL 1X PBS. 15 μL resuspended cells were transferred to 96-well flat bottom non-sterile black plates (Greiner Bio-one). 15 μL of the SYBR Green lysis buffer (2XSYBR Green, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100) was added to each well. Negative control (treated by DMSO) and positive controls (artesunate) were added to each set of experiments. Plates were incubated for 0.25 h at 37 °C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 497 and 520 nm respectively. The concentrations of compounds required to induce a 50% decrease in parasite growth (Ascorbic acid K1 IC_{50}) were calculated.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.011

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