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Original article

Antimalarial activity of new acridinone derivatives

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ABSTRACT

Malaria is one of the major threats concerning world public health. Resistance to the current antimalarial drugs has led to searches for new antimalarial compounds. Acridinone derivatives have recently demonstrated to be active against malaria parasite. We focused our attention on synthesized new acridinone derivatives, some of them resulting with high antiviral and trypanocidal activity. In this study new derivatives of 10-alyl-, 10-(3-methyl-2-butenyl)- and 10-(1,2-propadienyl)-9(10*H*)-acridinone were evaluated for their antimalarial activity against *Plasmodium falciparum*. To assess the selectivity, cytotoxicity was assessed in parallel against human MRC-5 cells. Inhibition of β-hematin formation was determined using a spectrophotometric assay. Mitochondrial bc_1 complexes were isolated from yeast and bovine heart cells to test acridinone inhibitory activity. This study resulted in the identification of three compounds with submicromolar efficacy against *P. falciparum* and without cytotoxic effects on human cellular line. One compound, **IIa** (1-fluoro-10-(3-methyl-2-butenyl)-9(10*H*)-acridinone), can be classified as hit for antimalarial drug development exhibiting IC_{50} less than 0.2 μg/mL with SI greater than 100. In molecular tests, no relevant inhibitory activity was obtained for our compounds. The mechanism of acridinones antimalarial action remains unclear.

1. Introduction

Malaria is one of the major parasitic diseases in the tropical and subtropical regions of the world and its aetiological agents are protozoans of the genus *Plasmodium*. *Plasmodium falciparum* is the most virulent among the four species infecting humans and is responsible for most of mortality. It is estimated that malaria causes nearly one million deaths, mostly of children under five years, and approximately 3.3 billion people, living in 109 countries, are presently at risk. Over 90% malaria deaths occur in Africa. In the Americas, 14% of the population is at risk; more than 2.5 million of malaria cases and 2593 deaths have been estimated for one year [1].

Currently, no single drug is effective for treating multidrug resistant malaria. Therapy based on the natural endoperoxide artemisinin or its semisynthetic derivatives combined with old drugs is the most appropriated alternative for an effective treatment delaying resistance appearing [2]. Artemisinin derivatives are fast-acting antimalarials [3] but on the basis of animal studies there remains concern with regard to potential neurotoxicity [4] and reproductive toxicity [5]. With the steady increase in the incidence of malaria worldwide together with its associated mortality and morbidity and the rapid emergence and spread of antimalarial drug resistance there is an urgent need for novel therapeutics.

In this way, Winter et al. [6] synthesized, tested and demonstrated the potent and selective in vitro antiplasmodial activity of haloalkoxyacridinones. On the other hand, these authors concluded that 10-*N*-substituted acridinones bearing alkyl side chains with tertiary amine groups at the terminal position represent efficacious chemosensitization pharmacophores [7].

In a recent publication [8], a unique approach toward developing such agents is disclosed. Quinolines act in the digestive vacuole of *P. falciparum* by inhibiting the metabolism of hemoglobin that the parasite needs for survival and causing the buildup of a toxic heme precursor. Resistance to the quinoline class of compounds is caused by mutations in the gene encoding *P. falciparum* chloroquine resistance transporter (PfCRT), a protein located in the digestive vacuole membrane that leads to reduced uptake of the drug in the food vacuole.

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The target of the quinoline remains sensitive to inhibition but the plasmodium organism escapes it by preventing access to the target. This type of resistance can be overcome by blocking the PfCRT transporter that is responsible for preventing the drug from accessing its target [9]. Taking advantage of this mechanism of resistance, the authors designed a series of compounds that are in essence multifunctional in that they consist of a central heme-targeting core to which are attached a chemosensitizing moiety and a group that enhances the partitioning of the drug into the acidic digestive vacuole. N-alkylamine acridinone derivative with chloro and 2-*N*,*N*-diethylalkyloxy as substituents on the acridinone core was obtained as the active agent. Walker [10] suggests that the last acridinone derivative could be used in combination with quinine as front line therapy to treat infection and prevent the development of resistance.

Taking into account these facts and our experience in the synthesis and characterization of acridinone core [11] we focus attention on new synthesized acridinones derivatives, some of them possessing high antiviral [12] and moderate trypanocidal activity [13]. In this paper we report the in vitro activity of 12 derivatives of 10-allyl-, 10-(3-methyl-2-butenyl)- and 10-(1,2-propadienyl)-9(10*H*)-acridinone, against *P. falciparum*. All compounds were examined in parallel for their cytotoxicity on human fibroblasts. Inhibition of heme polymerization and mitochondrial bc_1 complex activity were explored as possible mechanisms of antimalarial action.

2. Materials and methods

2.1. Reactives

All reagents purchased from commercial sources were used without further purification. Reactives used for chemical synthesis and analysis were obtained from Merck (Darmstadt, Germany). Dimethyl sulphoxide (DMSO), Malstat reagents, culture media and antimalarial drugs were from Sigma. Hemin (ferriprotoporphyrin IX chloride) was from Fluka Sigma-A.

2.2. Chemistry

The acridinones (**2a–d**) were obtained from the corresponding anilino benzoic acid (**1a–d**), using Ullman modifications in the presence of water as solvent and Higher Power Ultrasound, according to the literature [14]. The *N*-substituted acridinones derivatives (**Ia–d, IIa–d and IIIa–d**) were synthesized using a phase-transfer agent as we have previously reported [11]. The synthetic route is shown on the Fig. 1.

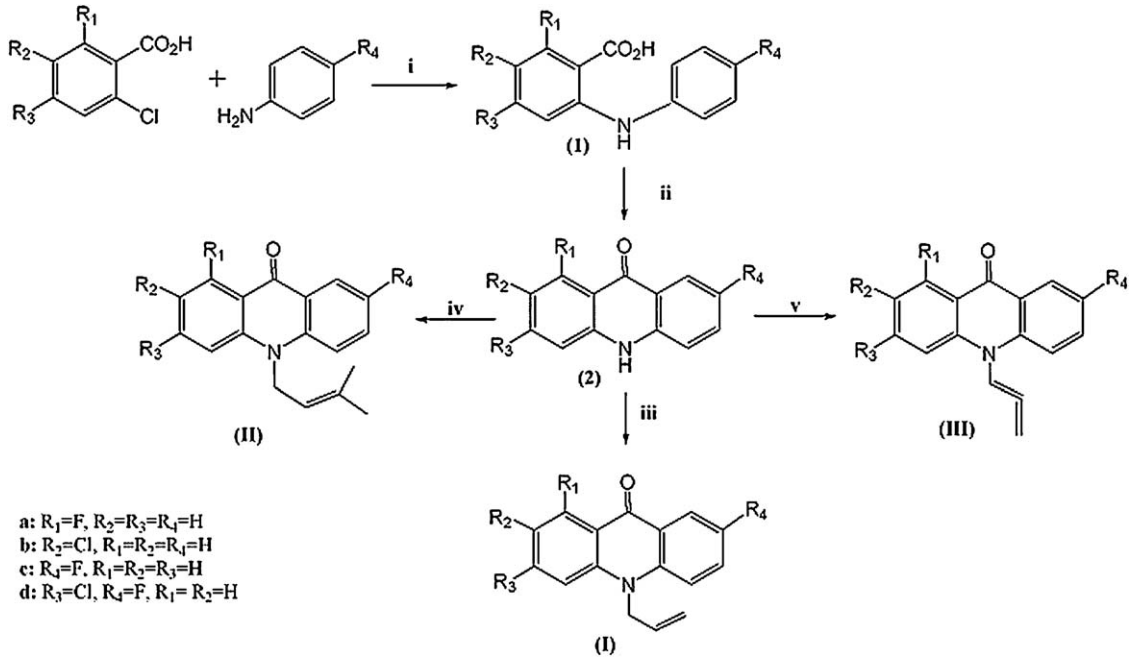
Melting points were measured on a Unimelt apparatus and were uncorrected. The 1H NMR spectra were recorded with a Bruker AC 200 Instrument, (Bruker Corp., Karlsruhe, Germany) at 200 MHz and the ^{13}CNMR spectra were recorded at 50 MHz for solutions in DCCl3 with tetramethylsilane as the internal standard. 2D NMR spectra were recorded with Bruker AM 500 Instrument at 500 MHz. Mass spectra were performed with a Shimadzu QP-5000 instrument (Shimadzu Corp., Tokyo, Japan) by electron impact ionization. Analysis (thin-layer chromatography) was performed on plates coated with silica gel G using appropriate eluents each time and warm sulphuric acid for detection.

2.3. Biological screening tests

Standard screening methodologies were adopted as have been described by Cos et al. [15]. Stock solutions of each compound were dissolved in 100% dimethyl sulphoxide (DMSO) at 20 mM and stored at 4 °C until use.

2.3.1. Test plate production

The experiments were performed in 96-well plates (Greiner), each plate containing all compounds at four-fold dilutions in a dose-titration range of 64 μM to 0.25 μM. Dilutions were carried



i. K_2CO_3/Cu/H_2O/ultrasonic 20 min; ii. H_2SO_4/100°C/2 h; iii. allyl bromide/KOH 50%/butanone/2.5 h; iv. 4.bromo-2-methyl-2-butene/KOH 50%/butanone/2.5 h; v. propargyl bromide/KOH 50%/ butanone/2.5 h.

Fig. 1. Scheme of acridinones synthetic route.

out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). All tests were run in duplicate.

2.3.2. Antiplasmodial activity

The chloroquine-susceptible *P. falciparum* GHA-strain was used. Parasites were cultured in human erythrocytes A^+ at 37 °C under a low oxygen atmosphere (3% O_2, 4% CO_2, and 93% N_2) in a modular incubation chamber. The culture medium was RPMI-1640, supplemented with 10% human serum. Two hundred microliters of infected human red blood cells suspension (1% parasitemia, 2% hematocrit) were added to each well of the plates with test compounds and incubated for 72 hours. After incubation, test plates were frozen at −20 °C. Parasite multiplication was measured by the Malstat method [16]. One hundred microliter of Malstat reagent were transferred in a new plate and mixed with 20 μL of the hemolysed parasite suspension for 15 minutes at room temperature. After addition of 20 μL NBT/PES solution and two hours incubation in the dark, the absorbance was spectrophotometrically read at 655 nm (Biorad 3550-UV microplate reader). Percentage growth inhibition was calculated compared to the negative blanks. Chloroquine sulphate and artemisinin were used as positive controls.

2.3.3. Cytotoxicity assay

Human fetal lung fibroblast (MRC-5 SV_2) cells, were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% FCS at 37 °C and 5% CO_2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the prediluted compounds and incubated at 37 °C and 5%CO_2 for 72 hours. Cytotoxicity was assessed fluorimetrically by adding resazurin for four hours at 37 °C. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm).

2.3.4. IC_{50}, CC_{50} and SI determination

Activities of compounds were expressed as inhibitory concentration 50%, i.e. the concentration of compound that inhibits 50% of microbial growth (IC_{50}) and human cell growth (CC_{50}). The values were calculated from the dose-response curves using the Statview™ software package. Selectivity index (SI) was calculated as follows: SI = CC_{50} (MRC-5)/IC_{50} (*P. falciparum*).

2.4. Inhibitory activity of β-hematin formation

Inhibition of β-hematin formation was developed in Eppendorf tubes mixing hemin and compounds at acidic environment using conditions of the Protocol Nº 3 proposed by Baelmans et al. [17] All acridinones were dissolved in DMSO, whereas, chloroquine diphosphate was prepared in distilled water and used as reference drug. Serial dilutions of all compounds were made, the doses range was from 1 to 20 molar equivalents to hemin (Meq) [18,19]. In control tubes either water or DMSO was added, the final concentration of DMSO per tube do not exceed 25% [19]. After 24 hours of incubation at 37 °C, the assay tubes were centrifuged for 15 min at 3300 *g*. Further processing was identical to that described by Baelmans et al. [17] for Protocol Nº 1. Finally, the β-hematin formed was dissolved in 0.1 M NaOH for spectroscopic quantitation. A 150 μL aliquot was transferred onto a 96-well plate and serial four-fold dilutions in 0.1 M NaOH were made. The amount of hematin was determined by measuring the absorbance at 405 nm using a microtiter plate reader. The drug concentration required inhibiting β-hematin formation 50% (IC_{50}) was determined.

2.5. Inhibitory activity on bc_1 complex

2.5.1. bc_1 complex isolation

The bc_1 complex isolated from *Saccharomyces cerevisiae* strain (DBY747) was used as surrogate of model organism. Yeast was grown until late log phase in autoclaved yeast extract, peptone, and dextrose (YPD) medium at 28–30 °C according to standard procedures [20] and it was harvested by differential centrifugation. The bc_1 complex was isolated from *S. cerevisiae* in analogy to the method described by Trembath and Tzagoloff [21] from about 2.5 kg pressed yeast. The isolation of cytochrome bc_1 complex from yeast mitochondria was carried out according to Geier et al. [22].

Bos taurus heart mitochondria was used to isolate cytochrome bc_1 as a mammalian system, fresh bovine hearts (1.2–1.5 kg) were homogenized followed by stepwise differential centrifugation [23]. The bc_1 complex was prepared following the methodology of Schägger et al. [24] modified as described by Gille et al. [25].

2.5.2. Inhibitory activity assay

Isolated bc_1 complexes were used to determine the activity of compounds. The bc_1 complex (6 nM) and acridinones, at final concentration of 250 μM, were diluted in 1 mL measurement buffer (250 mM Sacarosa, 0,2 mM EDTA, 50 mM KH_2PO_4, pH = 7,2) and 100 μmol/L cytochrome c. The enzymatic reaction started by addition of 75 μM decylubiquinol ($dUHQ_2$). The initial rate of the cytochrome c^{3+} reduction was calculated using an extinction coefficient of $\varepsilon_{550\text{-}540\ nm}$ = 19 $mM^{-1}cm^{-1}$. Triplicate measurements were performed and the results were expressed as percent of inhibition with respect to control experiments.

3. Results

Twelve compounds (Fig. 2) were tested for antimalarial and cytotoxic activity; the results are shown in Table 1. Derivatives of 10-(1,2-propadienyl)-9(10*H*)-acridinone (**III**) were inactive or marginally active showing IC_{50} values equal or higher than 64 μM, in contrast, three 10 allyl-9(10*H*)-acridinone derivatives exhibited antimalarial activity (IC_{50} < 64 μM). No cytotoxicity against MRC-5 fibroblast was detected for tested compounds. Three compounds (**Ib**, **Ie** and **IIa**) exhibited the most potent and selective antimalarial activity showing IC_{50} less than 0.5 μg/mL and SI greater than 39.

Inhibition of β-hematin formation test showed IC_{50} values over 20 Meq for all acridinones whereas chloroquine, the reference compound, exhibited IC_{50} of 1.2 ± 0.5 Meq.

Table 1
Activity of acridinones against *P. falciparum* and MRC-5 cells.

Compound No[a]	*P. falciparum*			MRC-5
	IC_{50} (μM)	IC_{50} (μg/mL) (< 0.2 μg/mL)[c]	SI[b] (> 100)[c]	CC_{50} (μM)
Ia	1.62	0.38	> 39.5	> 64
Ib	1.46	0.37	> 43.8	> 64
Ic	> 64			> 64
Id	37			> 64
IIa	0.57	0.16	> 112.2	> 64
IIb	> 64			> 64
IIc	> 64			> 64
IId	> 64			> 64
IIIa	64			> 64
IIIb	> 64			> 64
IIIc	64			> 64
IIId	> 64			> 64
Chloroquine	0.024			
Artemisinin	0.041			

[a] The structures can be found in Fig. 2.
[b] SI = CC_{50} (fibroblast)/IC_{50} (parasite).
[c] Activity/safety criteria for an antimalarial hit according to TDR. Nwaka et al. [28].

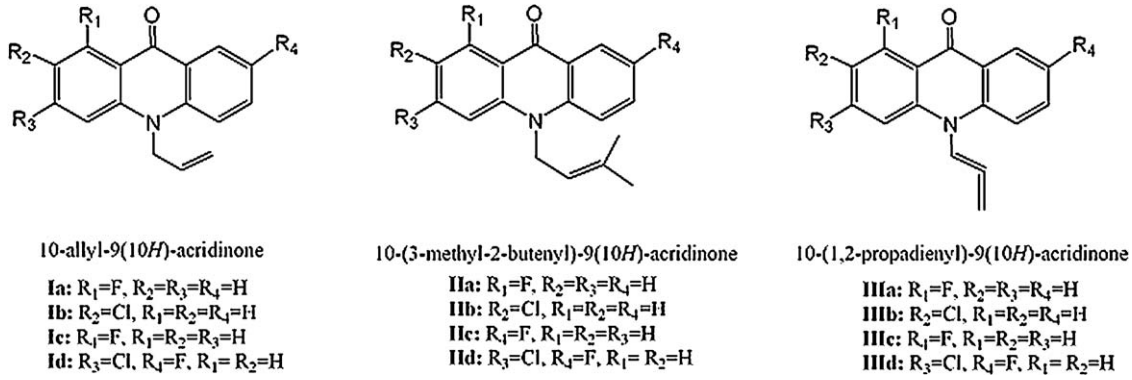


Fig. 2. Acridinones synthesized.

Table 2
Inhibition of bovine and yeast mitochondrial bc_1 complex activity by acridinone derivatives.

Compound N°	Inhibition (%) ± SD[a]	
	B. taurus	*S. cerevisiae*
Ia	81.9 ± 1.1	27.1 ± 3.2
Ib	64.7 ± 1.3	56.2 ± 10.6
Ic	60.7 ± 2.4	5.8 ± 1.4
Id	79.06 ± 2.9	75.0 ± 5.5
IIa	73.6 ± 1.8	19.7 ± 3.8
IIb	89.4 ± 2.3	84.5 ± 3.0
IIc	32.0 ± 0.4	5.4 ± 3.4
IId	37.3 ± 8.6	9.4 ± 3.4
IIIa	73.5 ± 1.6	41.1 ± 0.5
IIIb	50.6 ± 0.9	0
IIIc	60.1 ± 5.9	0
IIId	31.2 ± 7.15	0
Standards	IC_{50} (nM)	
	B. taurus	*S. cerevisiae*
Stigmatellin	1.1 ± 0.1	1.0 ± 0.1
Atovaquone	2104 ± 499	85 ± 3

[a] Inhibition was calculated at 250 μM for each acridinone.

The influence of compounds on bc_1 complex isolated from *S. cerevisiae* and *B. taurus* is shown in Table 2. At concentrations of 250 μM (about 500 times higher than the lowest IC_{50} in plasmodia) acridinones exhibited a moderate inhibition of the cyt bc_1 complex from bovine and yeast. However, there was no strong preferred inhibition of yeast or bovine cyt bc_1 complex. The compound **IIb** caused the higher inhibition in both systems, followed by **IIa**, **Ia**, **Id**, and **IIIa** compounds.

4. Discussion and conclusion

Several acridinone-based compounds have been studied for their potential as antimalarial agents [6,7,26,27]. In a recent report Kelly et al. [8] showed the potent activity of a 10-N- substituted acridinone which also act as chemosensitizer "verapamil-like".

The most active antimalarial acridinone already reported exhibited IC_{50} values against *P. falciparum* in the nanomolar [8] and picomolar range [6]. Our tested compounds exhibited weaker activity than those acridinones and the reference drugs (chloroquine and artemisinin). However, our compounds showed very low cytotoxicity against MRC-5 cell line ($CC_{50} > 64$ μM) and VERO cells, CC_{50} greater than 1000 μM [12]. Previously synthesized antimalarial 10-*N*-substituted acridinones exhibited higher cytotoxicity. The 10-*N*-substitute acridinones synthesized by Kelly et al. [7] exhibited IC_{50}s ranging from 17.6 to 59.5 μM on human granulocyte-macrophage colony whereas; dual function acridinones showed lower values (10.3 μM) against human foreskin fibroblast cells [8].

The WHO Special Programme for Research and Training in Tropical Diseases (TDR) defines for antimalarial "hit" an activity criterion to be IC_{50} less than 0.2 μg/mL with SI greater than 100 [28] and compound **IIa** (1-fluoro-10-(3-methyl-2-butenyl)-9(10*H*)-acridinone), a novel structure reported for first time in this study, met this requirement. Other experiments should be done to determine if this compound could be classified as a lead [28].

Our acridinones included different moieties at *N*-10 position (allyl, 3-methyl-2-buthenyl and 1,2-propadienyl), whereas, halogens (Chlorine and Fluorine) were alternative substituents at 1, 2 and 6 position on the acridinone cycles. Some relationship between the structures and antimalarial activity was apparent. We could observe that 10 allyl-9(10*H*)-derivatives are in general active structures. Similar results were obtained for antiviral evaluation [12] although different acridinones developed the highest activity. Besides, it seems apparent that fluorine at 1 position is an important structural feature inside on antimalarial activity. Potent antimalarial acridinones include fluorine in its structure; Winter et al. [6] showed that by increasing the number of fluorine atoms or the number of CF_3 substituents a further reduction in the IC_{50} value was observed. For antiviral activity 6-chloro-substitution seemed to be an important element [12]. 10-*N*-substituted acridinones obtained by Kelly et al. [7] showed antimalarial activity against a chloroquine-sensitive *P. falciparum* strain in the micromolar range (higher IC_{50} values than our 10-*N*-substitute acridinone). The inclusion of chlorine and 2-N,N-diethylalkyloxy strongly potentiated their antimalarial action [8].

Knowledge about active compounds mode of action is required to improve their medicinal activity. Heme interactions preventing hemozoin formation and mitochondrial complexes inhibition have been established as mechanisms of antimalarial acridinones [8,29]. As a preliminary evaluation of possible mechanism of antimalarial action, we tested acridinones inhibitory activity of β-hematin formation and mitochondrial bc_1 complex.

Various methods have been proposed to mimic in vitro the formation of malaria pigment. Assays detecting and measuring inhibition of β-hematin formation have been designed as indicator of antimalarial activity. As expected, our in vitro assay revealed a significant inhibitory activity for chloroquine, the obtained IC_{50} value was similar to those reported earlier [17–19]. For our acridinones, none inhibitory activity was observed at really high relative concentrations (20 Meq), suggesting they kill malaria parasite by a mechanism different of blocking hemozoin formation.

Some dihydroacridinediones have been reported to inhibit the malaria parasite respiratory pathway, causing a reduction in whole-cell O_2 consumption [30]. More recently, selective inhibition of the mitochondrial bc_1 complex by one related compound was demonstrated [29]. The acridinone **Id** caused a decrease in the mitochondrial transmembrane potential of *Trypanosoma cruzi* epimastigotes [13]. Here, inhibition of mitochondrial bc_1 complex activity was tested.

We analyzed the action of the compounds using *S. cerevisiae* as a surrogate of microorganism model and *B. taurus* as mammalian model. These systems were validated with stigmatellin (unspecific inhibitor) and atovaquone (specific inhibitor), obtaining IC_{50} values against bovine heart and yeast mitochondrial bc_1 complex similar to a previous report [29]. Selectivity indexes exhibited by reference drugs were also quite similar to those obtained using *B. taurus* and *P. falciparum* bc_1 complex [29].

No complete inhibition of mitochondrial complex was obtained even at compounds concentrations two thousands times higher than atovaquone IC_{50} values. Atovaquone exhibits similar values of activity in the isolated complex assay (3 nM) and cell-based assay (1 nM [29]). However, for our most active acridinones a shift between the isolated complex assay and protozoa-based assay of a factor more than 200 was observed. This shift could be due to off target activity.

Acridinones could inhibit the respiration due to their action on mitochondrial bc_1 complex. However, we observed similar activity on both mammalian and yeast system. These results suggest that compounds act on bc_1 complex, but their action is unspecific in the molecular model. The selectivity index of active compounds for cellular system was greater than 30; while for bc_1 complex was less than one.

The unspecific action of acridinones on the bc_1 complex in contrast to the specific action of acridinediones [29] suggests that the dione structural motive in acredinediones is essential for selectivity at this target. Therefore, it is likely that damages by acridinones in parasites are caused by mechanisms different from mitochondrial bc_1 inhibition. However, since inhibition of mammalian bc_1 complex by acridinones at higher concentrations was also observed, this could be of toxicological relevance.

In conclusion, we confirmed the antimalarial potential of acridinone-based compounds. A "hit" for antimalarial drug discovery was obtained. Work is underway to elucidate the mechanism of its antiplasmodial action and to evaluate in vivo efficacy in murine models of malaria.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Ethical approval: Not required.

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