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Antimalarial acridines: Synthesis, in vitro activity against *P. falciparum* and interaction with hematin

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ABSTRACT

A series of acridine derivatives were synthesised and their in vitro antimalarial activity was evaluated against one chloroquine-susceptible strain (3D7) and three chloroquine-resistant strains (W2, Bre1 and FCR3) of *Plasmodium falciparum*. Structure–activity relationship showed that two positives charges as well as 6-chloro and 2-methoxy substituents on the acridine ring were required to exert a good antimalarial activity. The best compounds possessing these features inhibited the growth of the chloroquine-susceptible strain with an $IC_{50} \leqslant 0.07$ μM, close to that of chloroquine itself, and that of the three chloroquine-resistant strains better than chloroquine with $IC_{50} \leqslant 0.3$ μM. These acridine derivatives inhibited the formation of β-hematin, suggesting that, like CQ, they act on the haem crystallization process. Finally, in vitro cytotoxicity was also evaluated upon human KB cells, which showed that one of them 9-(6-ammonioethylamino)-6-chloro-2-methoxyacridinium dichloride **1** displayed a promising antimalarial activity in vitro with a quite good selectivity index versus mammalian cell on the CQ-susceptible strain and promising selectivity on other strains.

1. Introduction

Malaria is the third cause of death per infectious disease after tuberculosis and AIDS as it affects 200–500 million people and causes 1–2 million fatalities every year.[1] Quinine, and then chloroquine (CQ, Chart 1) were employed in the past five decades to fight malaria not only because of their ease of use and low cost, but also because they presented very few side effects. Unfortunately resistant strains of *Plasmodium falciparum* have appeared, however resistance mechanisms seem to involve CQ itself rather than a modification in the drug targets,[2] as it is explained in the following paragraph.

A new class of antimalarial agents, acridines, and especially 9-aminoacridines (quinacrine and 9-aminoacridines), has been developed in the last 15 years.[3,4] In addition, a particularly promising strategy for optimising their antimalarial activity has been recently used, that involves the synthesis of 6-chloro-2-methoxyacridine derivatives.[5–7] 9-Aminoacridines and quinolines (like CQ) are known to be potent DNA intercalators[8] and can inhibit DNA transcription in parasites.[9–11] However, it is now commonly admitted

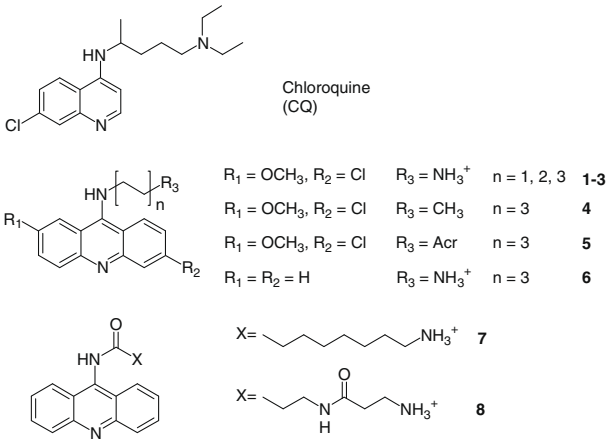

Chart 1. Chloroquine and acridine derivatives tested in this work.

Abbreviations: BOP, benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate; CDI, carbonyldiimidazole; CQ, chloroquine; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid.

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that the main reason for which they are toxic for the parasite stands in the fact that they interfere with the crystallization of free haem generated during the degradation of hemoglobin.[12–16] This constitutes a particularly interesting property since the haem detoxification pathway does not seem to be dependent on any specific enzyme, which, consequently, makes the appearance of resistance harder. Thus, CQ has been extensively used for 20 years before the emergence of resistance, whereas one year was sufficient for the parasite to develop resistance against pyrimethamine (inhibitor of dihydropteroate synthase, an enzyme involved in the metabolism of folate).[17] Two other arguments also lead to consider that the haem detoxification pathway still represents a valid target for the discovery of new anti-malarial drugs. First, in the haem detoxification pathway, the 'drug receptor' is free haem that doesn't exist at such a concentration under physiological conditions in human cells. It then represents a better target in comparison to some parasite enzymes that are analogous to those found in human (various proteases, farnesyl transferase and enzymes involved in choline uptake). Second, the haem detoxification pathway is not by itself directly involved in quinoline resistance, whereas drug transporters (PfCRT) are through their involvement in the alteration of drug accumulation into the parasite which results from a reduced uptake of the drug, an increased efflux, or a combination of the two processes.[18] In fact, for most of resistant Pf strains, this transporter gene (*pfcrt*) present point mutations leading to acidification of the digestive vacuole and efflux of CQ.

In a previous paper,[7] we described the synthesis and the in vitro antimalarial activity of a series of 9-substituted acridine derivatives. Some of them showed significant antimalarial activities against CQ-resistant strains with IC_{50} values $\leqslant$0.20 μM. In this paper, we report the synthesis and the in vitro antimalarial activity of new molecules containing the same scaffold (i.e., 9-aminoacridine). These compounds are listed in Chart 1. Two novel shortened chain analogues (compounds **1** and **2**) of 9-(6-ammoniohexylamino)-6-chloro-2-methoxyacridinium dichloride, the more efficient formerly studied compound (compound **3**) were synthesised. We expected them to be more potent than CQ against CQ-resistant strains, as it was already observed in the case of 4-aminoquinoline analogues of CQ with shortened alkylamino side chains.[19,20] A 9-heptylaminoacridine (compound **4**) was also synthesised and its antimalarial activity was compared to that of compound **3**, in order to evaluate the role of the terminal positive charge of 9-amino-substituent. One bis-acridine derivative **5**, whose anti-malarial activity was already described in the literature,[5] was included in this study. It will be useful to evaluate the contribution of two protonated acridine rings. Compound **6** differs from **3** only by the absence of 6-chloro and 2-methoxy substituents on the acridine ring. Finally, two 9-amido acridine derivatives (compounds **7** and **8**) whose study will be helpful to assess if a positive charge on the acridine ring is necessary to confer a significant antimalarial activity, were synthesised. Among all the compounds tested, compounds **4** and **8** are new acridine derivatives never published to our knowledge. The synthesis of the other ones was already described (**1–3**,[21,22] **6**[23]), but their antimalarial activities were never evaluated except for the compound **7**[7] and the bis-acridine derivative **5**.[5] In addition, experiments were performed to correlate the antimalarial activity of molecules **1–8** with their ability to interact with hematin and prevent the crystallization of free haem.

2. Chemistry

The 9-amino-6-chloro-2-methoxyacridine derivatives **1**–**5** were prepared from commercial 6,9-dichloro-2-methoxyacridine according to already described procedures.[7,22] Addition of an excess of diaminoalkane afforded the 9-amino substituted acridine derivatives **1**–**4**, while the bis-acridine derivative **5** was obtained by modifying the relative proportion of 1,6-diaminohexane (0.5 equiv with respect to acridine) (Scheme 1).

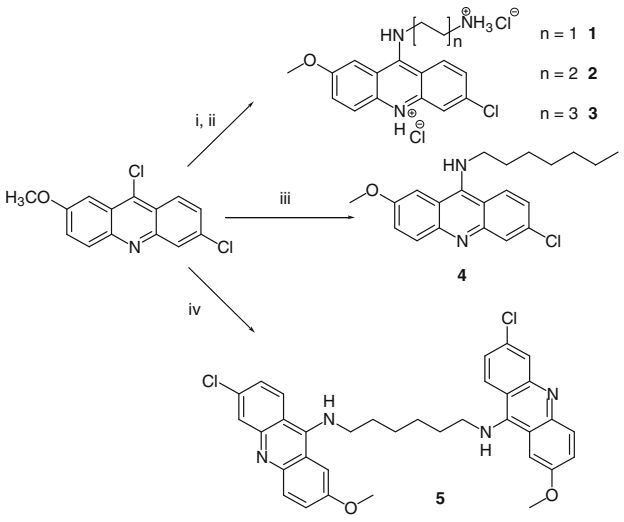
Surprisingly, addition of an excess of 1,6-diaminohexane to 9-chloroacridine, yielded the bis-acridine compound as major product. Consequently, the protection of one of the amino substituents of 1,6-diaminohexane was necessary and the acridine derivative **6** was obtained in two steps, using mono *tert*-butoxycarbonyl-diaminohexane, followed by classical TFA treatment to remove the protecting group (Scheme 2a). Two 9-amido acridine derivatives were also synthesised and studied (Scheme 2b). The acridine derivative **7** was synthesised with the workup procedure reported earlier from our laboratory.[7] The acridine derivative **8** was prepared by two successive peptidic coupling reactions using β-alanine units protected by a *tert*-butoxycarbonyl group.

Both elemental analysis and spectral data (^1H and ^{13}C NMR, UV–vis, ES MS, HRMS, or microanalysis) of the synthesised compounds are in agreement with the structures of the products.

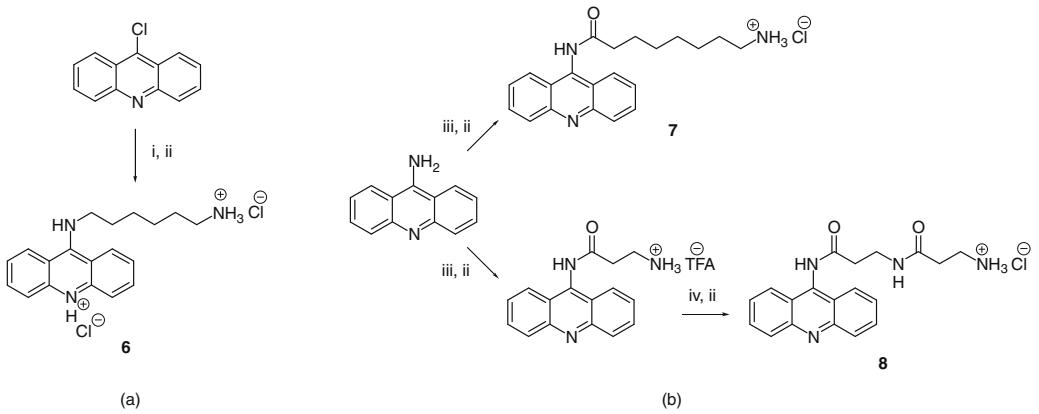
3. Antimalarial activity, interaction with hematin and cytotoxicity

The antimalarial activity was tested on one CQ-susceptible *P. falciparum* strain 3D7 (Africa), and three CQ-resistant strains W2 (Indochina), FCR3 (the Gambia) and Bre1 (Brazil). The antimalarial activity was quantified from the inhibition of parasite growth. Results are reported as the micromolar inhibitory concentrations necessary to reduce ^3H-hypoxanthine incorporation by 50% (IC_{50}, Table 1).

Interactions with the haem detoxification pathway were evaluated by different approaches. β-Hematin crystallization inhibition assays were performed using a slightly modified procedure of the β-hematin crystallization inhibitory activity (BHIA) assay, described by Parapini et al.[24] The experimental conditions used (hemin, DMSO, pH 5) afford the optimal reaction to detect π–π interactions between haem and drug. The acetate buffer maintains the pH of the reaction in the appropriate range as pH 5 corresponds to the isoelectric point of hematin and allows the best π–π complexation between hematin and aromatic compounds like acridines. It is noteworthy that this pH coincides also with the estimated pH of the parasite food vacuole.[25] Results are reported as the concentration that is necessary to reduce hematin crystallization by 50% (IC_{50}).

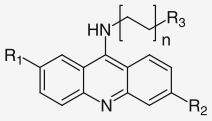
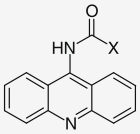
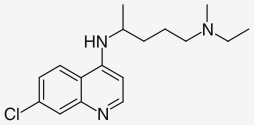


Scheme 1. Synthesis of compounds **1**–**5**. Reagents and conditions: (i) H_2N–$(CH_2)_n$–NH_2, 80 °C, 4 h; (ii) TFA/CH_2Cl_2 1:1, rt, 16 h, then DOWEX Cl^-; (iii) *n*-heptylamine, 80 °C, 4 h; (iv) 1,6-hexanediamine, DIEA, DMF, 80 °C, 16 h.



Scheme 2. Synthesis of compounds **6**–**8**. Reagents and conditions: (i) H_2N–$(CH_2)_n$–NHBoc, DIEA, 60–80 °C, 6 h; (ii) TFA/CH_2Cl_2 1:1, rt, 16 h, then DOWEX Cl^- (no DOWEX for **8** synthesis); (iii) BOP, DIEA, DMF, rt (50 °C for **8** synthesis), 16 h; (iv) CDI, DMF, rt, 16 h.

Table 1
Comparative in vitro efficiency of chloroquine (CQ) and the acridine derivatives against chloroquine-susceptible and chloroquine-resistant *P. falciparum* strains

Compound		*Plasmodium falciparum* strains			
		Chloroquine-susceptible		Chloroquine-resistant	
		3D7 IC_{50} (μM)	W2 IC_{50} (μM)	FCR3 IC_{50} (μM)	Bre1 IC_{50} (μM)
R_1 = OCH_3 R_2 = Cl R_3 = NH_3^+, n = 1	**1**	0.042 ± 0.019	0.25 ± 0.03	0.33 ± 0.06	0.28 ± 0.05
R_1 = OCH_3 R_2 = Cl R_3 = NH_3^+, n = 2	**2**	0.067 ± 0.012	0.25 ± 0.04	0.25 ± 0.02	0.30 ± 0.06
R_1 = OCH_3 R_2 = Cl R_3 = NH_3^+, n = 3	**3**	0.13 ± 0.04	0.18 ± 0.05	0.20 ± 0.02	0.17 ± 0.04
R_1 = OCH_3 R_2 = Cl R_3 = CH_3, n = 3	**4**	18.1 ± 6.3	27.2 ± 9.0	14.6 ± 5.8	16.3 ± 6.7
R_1 = OCH_3 R_2 = Cl R_3 = Acr, n = 3	**5**	3.39 ± 0.60	4.55 ± 0.85	1.01 ± 0.59	1.11 ± 0.69
R_1 = R_2 = H R_3 = NH_3^+, n = 1	**6**	1.13 ± 0.25	0.60 ± 0.2	0.36 ± 0.01	0.43 ± 0.10
					
X = $(CH_2)_7NH_3^+$	**7**	41.9 ± 10.1	50.0 ± 2.2	28.6 ± 7.9	26.7 ± 5.7
X = $(CH_2)_2NHCO(CH_2)_2NH_3^+$	**8**	40.9 ± 9.5	20.9 ± 4.3	17.8 ± 6.0	21.6 ± 7.9
 CQ		0.018 ± 0.002	0.44 ± 0.06	0.50 ± 0.11	0.52 ± 0.10

The stoichiometry and the dissociation constant K_d of the hematin/acridine complex, as well as the pK_a of the acridine moieties were also determined, using spectrophotometric methods in both cases. All these results are presented in Table 2.

In vitro cytotoxicity of the synthesised compounds was also evaluated upon human KB cells, a cell line derived from a human carcinoma of the nasopharynx system and expressed as the micromolar concentrations (IC_{50}) causing 50% of cell death (Table 3).

4. Results and discussion

Antimalarial activities are summarised in Table 1. Compounds **1** and **2** display an antiplasmodial potency of the same order of magnitude than CQ on CQ-susceptible strain 3D7, as they show IC_{50} values in the submicromolar range (42 nM for **1** and 67 nM for **2**). These values are lower than that displayed by **3** on this strain (130 nM). The antimalarial activity tested on CQ-resistant strains also shows that three compounds (**1**–**3**) are more active than chloroquine; a fourth compound, **6**, is also more efficient than CQ on FCR3 and Bre1 strains. Replacement of the 6-chloro and 2-methoxy substituents by an hydrogen atom (acridine derivatives **6** vs **3**) decreases the inhibitory potency by threefold on CQ-resistant strains and by ninefold on CQ-susceptible strain 3D7. A larger decrease of antimalarial activity is observed when the terminal positive charge on the 9-side chain is removed. Indeed, depending upon the strains, **4** is 73–151-fold less potent than **3**. The antima-

Table 2
Biophysical data: pK_a and interaction with hematin

Compound	pK_a[a]	BHIA	Complex [hematin]/[drug]	
		IC_{50} (mM)	Stoichiometry	K_d (μM)
CQ	8.10	0.9	2:1	5.4
1	7.94	4.6	1:1	4.2
2	9.10	1.0	3:2	8.1
3	9.20	0.4	3:2	4.4
4	7.50	1.5	3:2	5.4
5	8.23	1.3	1:1	20
6	9.86	0.3	3:2	4.6
7	4.59	1.1	nd	nd
8	4.53	1.8	3:2	4.0

[a] pK_a values were determined using spectroscopic methods in aqueous medium, 150 mM NaCl. All compounds were dissolved before use either in water or DMSO.

Table 3
In vitro cytotoxicity and selectivity index

Compound	IC_{50} KB (μM)	Selectivity Index (SI)			
		3D7	W2	FCR3	Bre1
CQ	22.5[a]	1250	51	45	43
1	2.07[a]	50	8	6	7
2	0.60[b]	8.9	2.4	2.4	2
3	0.70[b]	5.4	3.9	3.5	4.1
4	0.85[b]	0.04	0.02	0.06	0.05
5	0.05[b]	0.3	0.01	0.05	0.045
6	0.79[a]	0.7	1.3	2.2	1.8
7	52.9[a]	1.3	1.1	1.8	2.0
8	60.1[a]	1.5	2.9	3.4	2.8

SI was calculated as the ratio of IC_{50} for in vitro cytotoxicity on KB to the IC_{50} of plasmodial inhibition.
[a] IC_{50} values determined in duplicate.
[b] IC_{50} values determined in triplicate.

larial activity is however partially restored when a second positively charged acridine is grafted (**5**). However, as can be seen from Table 1, the IC_{50} values measured for bis-acridine **5** are ranging from 1.01 μM to 4.55 μM, showing a strong variability between different CQ-resistant Plasmodium strains, and are rather disappointing when compared to the values previously published by Girault et al.[5] Taken altogether, those first results on compounds **1–6** then suggest that both the presence of the chloro and methoxy substituents on the acridine ring and that of two positive charges at physiological conditions are required to get a good antimalarial activity.

An even more important loss of inhibitory potential is observed when the only positive charge is located on the side chain with a neutral acridine moiety (compound **7** vs compound **3**) with IC_{50} values 160–320-fold higher in this case. Such a loss of antiplasmodial effect was already observed for CQ analogues, and it was also shown that chemical modifications that imply the loss of the quinoline positive charge beared by the heterocycle of CQ tended to have a more pronounced impact on antimalarial activity than did modifications of the side chain.[26,27] By contrast to CQ analogues, acridine compounds display here similar potency against CQ-resistant strains when the only positive charge is located either on the acridine nucleus (**4**) or at the end of the side chain (9-amidoacridine **8**). Those later results on compounds **7** and **8** then confirm those obtained with compounds **1–6**, and, in summary, the results of Table 1 clearly indicate that both the presence of 6-chloro and 2-methoxy substituents on the acridine ring and that of two positive charges, one on the acridine ring and one on the terminal function of the side chain, are required for an optimal anti-malarial activity.

The inhibition of hematin crystallization was evaluated for all the compounds and the IC_{50} values measured by the BHIA method as well as the stoichiometry and the K_d value for the drug/hematin complexes are reported in Table 2. First, The IC_{50} values were in the same order of magnitude (0.3–4.6 mM) as the one exhibited by CQ (0.9 mM), the reference compound, which suggested that this class of compounds also acted on haem crystallization. Second, the ratio for the stabilized complex between hematin and most of the acridine derivatives tested under these conditions (**2–4** and **6**) was found to be 3:2, which is close to that obtained for CQ (2:1). A 1:1 ratio was obtained with the bis-acridine derivative **5**; which could be explained in this case by the fact that one hematin may be stacked between the two acridine moieties. The same ratio was also found for the shortest acridine derivative **1**.

The characterisation of all hematin/drug complexes give access to K_d values, which are all in the same range (from 4.2 to 8.1 μM), except for compound **5** (K_d = 20 μM). Those discrepancies in K_d and stoichiometry values obtained for the bis-acridine derivative **5** (compared to CQ and the other compounds) are likely due to the second acridine moiety, but unexpectedly this second protonated acridine does not improve the affinity for hematin. Similar K_d and hematin crystallization IC_{50} values were obtained when the 6-chloro and 2-methoxy substituents are replaced by hydrogen (acridine derivatives **6** vs **3**). However, acridine derivative **6** has a lower anti-malarial activity than **3** (threefold on CQ-resistant strains and up to ninefold on CQ-susceptible strain 3D7, Table 1), suggesting that in this particular case the antiplasmodial potency could not be entirely explained by the interaction with hematin. Indeed, the protonation state of **6** (pK_a = 9.86) may affect its ability to pass through the membrane of the food vacuole, which could explain the difference of antimalarial activity, at least on CQ-susceptible strains.

The best inhibition of hematin crystallization is obtained with compounds that bear one positive charge on the acridine moiety (whatever the substitution on it), and one positive charge on the side chain at pH 5, the distance between those two charges being also a significant factor. Indeed, the highest inhibition of hematin crystallization is found with the compound bearing the hexyl chain (IC_{50} = 0.4 and 0.3 mM, respectively, for acridine derivatives **3** and **6**), whereas the 6-ammoniobutylamino-acridine derivative **2** and the bis-acridine compound **5** display a lower inhibition comparable to CQ (IC_{50} = 0.9–1.3 mM). A lower inhibition is also observed when there is only one positive charge (IC_{50} = 1.5 and 1.8 mM, respectively, for compounds **4** and **8**). Strikingly, one of the most potent compounds on parasite (acridine derivative **1**) showed the weakest hematin crystallization inhibition (IC_{50} = 4.6 mM). Nevertheless, acridine derivative **1** displays a pK_a value similar to that of CQ, it will thus be trapped like CQ, once inside acidic compartments (i.e., food vacuole).[28] This behaviour may explain the good antimalarial potency of this compound on the CQ-susceptible strain 3D7.

The comparison of the various characteristics of the hematin/drug complexes described in Table 2 then suggests that the main interaction between hematin and the various drugs occurs between the acridine ring and the porphyrin ring system, which could be hydrophobic as well as electrostatic.[29,30] This has been confirmed by the UV–vis spectra (data not shown) that display significant hypochromic shifts (−40%). Indeed, such hypochromicity values are comparable with those obtained in the case of porphyrin interactions with DNA,[31,32] giving evidence for π–π stacking interactions. In addition, the optimal chain length grafted on the 9-position appears to be a hexyl chain which suggests that an electrostatic interaction may occur between the haem propionate groups and the terminal ammonium group of the acridine derivatives **3** and **6**.

In vitro cytotoxicity was assessed by using KB cells and the selectivity index (SI) calculated as the ratio of IC_{50} for in vitro cytotoxicity on KB cells to the IC_{50} of plasmodial inhibition on CQ-susceptible and -resistant parasites.[33,34] The results are presented in Table 3. Among the most active compounds on *P. falciparum*, compound **1** exhibits a low toxicity against KB cells line, with a selectivity index ranging from 7 for CQ-resistant strains to 50 for the

CQ-susceptible strain 3D7. Acridine derivative **2**, whose antimalarial activity is similar to **1**, displays more toxicity and therefore less selectivity towards plasmodia, with a selectivity index ranging between 2 and 9. Selectivity indexes are comparable for the compound **3**, whatever the strains; acridine derivatives **6**, **7** and **8** show no selectivity at all and compounds **4** and **5** show even more cytotoxic properties than antimalarial activity.

5. Conclusion

In summary, convenient, affordable and rapid syntheses were used to obtain potent antimalarials with aminoacridine as scaffold. Structure–activity relationship showed that under physiological conditions two positives charges, one on the acridine ring and the other at the end of a 9-(6-ammonioalkylamino) chain were required to exert a good antimalarial activity as well as 6-chloro and 2-methoxy substituents on the acridine ring. Experiments carried out with hematin suggest that these compounds likely act by inhibition of the haem crystallization process. Indeed, all of the acridine derivatives described here are able to interact with hematin but different hematin/drug complexes are obtained with structurally close compounds, which is still not fully understood. Besides, even if all of the efficient compounds are able to inhibit haem crystallization, no tight correlation was found between antimalarial activity and hematin crystallization IC_{50} values. Nonetheless, the acridine derivative **3**, the most potent compound on CQ-resistant strains, is the best inhibitor of haem crystallization.

Finally, among the compounds presented, **1** displayed promising antimalarial activity in vitro against *P.* falciparum with quite good selectivity index versus mammalian cell on the CQ-susceptible strain (3D7) and promising selectivity on other strains (W2, FCR3, Bre1) that could be optimised on the basis of further SAR studies. Future work will focus on this compound which could be a lead for further structural modifications.

6. Materials and methods

6.1. Chemistry

Most of the reactions were carried out in the dark. All other solvents and reagents were pure grade and used without purification. The following commercially available chemicals were pure grade and used as received: 9-aminoacridine, ethylene diamine, 1,4-diaminobutane and *n*-heptylamine (Acros), 6,9-dichloro-2-methoxyacridine and 1,6-hexanediamine (Aldrich). Synthesis of 9-(6-ammoniohexylamino)-6-chloro-2-methoxyacridinium dichloride **3** was reported earlier from our laboratory.[7]

Reactions were monitored by thin layer chromatography (TLC) performed on silica gel sheets containing UV fluorescent indicator (60 F254 Merck). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 200, Bruker AC 250, Bruker AV 360 and Bruker AC 400 spectrometers (200, 250 and 400 MHz for ^{1}H, respectively, and 50, 63, 90 and 100 MHz for ^{13}C, respectively). Chemical shifts, δ, are reported in ppm taking residual $CDCl_3$ or CD_3OD as the reference. Mass spectra were recorded on a Finningan-MAT-95-S, using $MeOH/CH_2Cl_2/H_2O$ (45:40:15, v/v) as solvent. High-resolution mass spectrometry analyses were performed by the 'Service de Spectrométrie de Masse de l'ICSN' Gif-sur-Yvette. Elemental analyses were performed by the Service Central de Microanalyse du CNRS de Gif-sur-Yvette.

6.1.1. 9-(6-Ammonioethylamino)-6-chloro-2-methoxyacridinium dichloride (1)

This compound was synthesised from 6,9-dichloro-2-methoxyacridine (1.2 g, 4.30 mmol) and ethanediamine (20 mL), according to an already described procedure[22] and obtained in 92% yield. 75 mg of N^1-(6-chloro-2-methoxy-acridin-9-yl)-ethane-1,2-diamine was then dissolved in TFA (2 mL) and stirred overnight at room temperature. TFA was then evaporated and the solid was dissolved in distilled water and passed over an ion exchange resin Dowex Cl^-. The chloride salt, 9-(6-ammonioethylamino)-6-chloro-2-methoxyacridinium dichloride **1**, was obtained quantitatively, after lyophilization.

^{1}H NMR (CD_3OD, 250 MHz) δ ppm: 2.98 (t, J = 6.50 Hz, 2H–CH_2 2), 3.75 (t, J = 6.50 Hz, 2H–CH_2 1), 3.93 (s, 3H, O–CH_3), 7.20 (dd, J_1 = 9.50 Hz, J_2 = 2.16 Hz, 1H–CH Acr 7′), 7.33 (dd, J_1 = 9.50 Hz, J_2 = 2.88 Hz, 1H–CH Acr 3′), 7.38 (d, J = 2.88 Hz, 1H–CH Acr 1′), 7.74 (d, J = 9.50 Hz, CH Acr 4′), 7.78 (d, J = 2.16 Hz, 1H–CH Acr 4′), 8.12 (d, J = 9.50 Hz, CH Acr 8′).

^{13}C NMR (CD_3OD, 63 MHz) δ ppm: 43.2 (CH_2 2), 53.1 (CH_2 1), 56.1 (O–CH_3), 100.8 (CH Acr), 116.0 (C Acr), 118.6 (C Acr), 124.4 (CH Acr), 126.0 (CH Acr), 126.7 (CH Acr), 126.9 (CH Acr), 130.2 (CH Acr), 136.3 (C Acr), 146.7 (C Acr), 148.9 (C Acr), 152.5 (C Acr), 157.2 (C Acr).

MS (ES) m/z: 302.1 (MH^+) (100%).

HRMS calcd for $C_{16}H_{16}ClN_3O$ (MH^+): 302.1060, found: 302.1050.

UV–vis (H_2O): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 274 (38 900), 349 (4090), 422 (6 540), 442 (7 330).

6.1.2. 9-(6-Ammoniobutylamino)-6-chloro-2-methoxyacridinium dichloride (2)

This compound was synthesised from 6,9-dichloro-2-methoxyacridine (1.0 g, 3.59 mmol) and 1,4-butanediamine (20 mL), according to an already described procedure[22] and obtained in 93% yield. 75 mg of N^1-(6-chloro-2-methoxy-acridin-9-yl)-butane-1,2-diamine was then dissolved in TFA (2 mL). The mixture was stirred overnight at room temperature, then TFA was evaporated. The solid was dissolved in distilled water and passed over an ion exchange resin Dowex Cl^-. The chloride salt, 9-(6-ammoniobutylamino)-6-chloro-2-methoxyacridinium dichloride **2**, was obtained quantitatively, after lyophilization.

^{1}H NMR (CD_3OD, 360 MHz) δ ppm: 1.55 (qn, J = 7.40 Hz, 2H–CH_2 3), 1.82 (qn, J = 7.40 Hz, 2H–CH_2 2), 2.65 (t, J = 7.40 Hz, 2H–CH_2 4), 3.84 (t, J = 7.40 Hz, 2H–CH_2 1), 3.97 (s, 3H, O–CH_3), 7.29 (dd, J_1 = 9.36 Hz, J_2 = 2.16 Hz, 1H–CH Acr 7′), 7.42 (dd, J_1 = 9.36 Hz, J_2 = 2.88 Hz, 1H–CH Acr 3′), 7.53 (d, J = 2.88 Hz, 1H–CH Acr 1′), 7.82 (d, J = 9.36 Hz, CH Acr 4′), 7.86 (d, J = 2.16 Hz, 1H–CH Acr 4′), 8.27 (d, J = 9.36 Hz, CH Acr 8′).

^{13}C NMR (CD_3OD, 63 MHz) δ ppm: 30.1 (CH_2), 29.5 (CH_2), 30.5 (CH_2), 41.9 (CH_2), 56.2 (O–CH_3), 101.2 (CH Acr), 114.9 (C Acr), 115.8 (C Acr), 118.4 (C Acr), 124.2 (CH Acr), 126.0 (CH Acr), 127.0 (CH Acr), 127.0 (CH Acr), 130.3 (CH Acr), 136.4 (C Acr), 149.2 (C Acr), 152.8 (C Acr), 157.2 (C Acr).

MS (ES) m/z: 329.1 (MH^+) (100%).

HRMS calcd for $C_{18}H_{20}ClN_3O$ (MH^+): 330.1373, found: 330.1369.

UV–vis (H_2O): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 277 (17 800), 341 (4 060), 422 (5380), 442 (4760).

6.1.3. N^1-(6-Chloro-2-methoxy-acridin-9-yl)-heptylamine (4)

A mixture of 6,9-dichloro-2-methoxyacridine (1 g, 3.59 mmol) and heptylamine (3.75 equiv, 20 mL) was heated at 80 °C for 4 h. The mixture was diluted with CH_2Cl_2, washed with water and dried over Na_2SO_4. After filtration and evaporation, the crude product was purified by chromatography over silica gel with CH_2Cl_2/CH_3OH (99:1 to 96:4, v/v) to afford N^1-(6-chloro-2-methoxy-acridin-9-yl)-heptylamine **4** in 61% yield (780 mg, 2.18 mmol).

^{1}H NMR (CD_3OD, 360 MHz) δ ppm: 0.82 (t, J = 6.84 Hz, 3H–CH_3 7), 1.20 (m, 4H–CH_2 5 + 6), 1.29 (m, 4H–CH_2 3 + 4), 1.73 (qn, J = 6.84 Hz, 2H–CH_2 2), 3.73 (t, J = 6.84 Hz, 2H–CH_2 1), 3.93 (s, 3H–O–CH_3), 7.22 (dd, J_1 = 9.36 Hz, J_2 = 2.16 Hz, 1H–CH Acr 7′), 7.36 (dd, J_1 = 9.36 Hz, J_2 = 2.88 Hz, 1H–CH Acr 3′), 7.44 (d,

J = 2.88 Hz, 1H–CH Acr 1′), 7.78 (d, J = 9.36 Hz, CH Acr 4′), 7.82 (d, J = 2.16 Hz, 1H–CH Acr 4′), 8.16 (d, J = 9.36 Hz, CH Acr 8′).

^{13}C NMR (CD_3OD, 90 MHz) δ ppm: 14.2 (CH_3), 22.9 (CH_2), 27.3 (CH_2), 29.4 (CH_2), 31.7 (CH_2), 32.1 (CH_2), 55.8 (O–CH_3), 100.4 (CH Acr), 114.8 (C Acr), 117.3 (C Acr), 124.0 (CH Acr), 125.4 (CH Acr), 125.6 (CH Acr), 126.0 (CH Acr), 128.2 (C Acr), 136.1 (C Acr), 145.2 (C Acr), 147.6 (C Acr), 152.5 (C Acr), 156.3 (C Acr).

MS (ES) m/z: 357.2 (MH^+) (100%).

Anal. Calcd for $C_{21}H_{25}ClN_2O{\cdot}0.5H_2O{\cdot}0.2CH_2Cl_2$: C, 66.50; H, 6.95; N, 7.32. Found: C, 66.52; H, 7.03; N, 7.24. MM = 392.5.

UV–vis (DMSO): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 286 (14 500), 345 (1 190), 426 (3 070), 450 (2 500).

6.1.4. *N*,*N*^{1}-Bis-(6-chloro-2-methoxy-acridin-9-yl)-hexane-1,6-diamine (5)

A solution of 6,9-dichloro-2-methoxyacridine (1.66 mmol, 460 mg), 1,6-hexanediamine (0.83 mmol, 97 mg) and DIEA (1.66 mmol, 274 μL) was heated at 80 °C in DMF for 16 h. After concentration under reduced pressure, water was added with stirring. The resulting solid was filtered and purified by MPLC with CH_2Cl_2/MeOH (5–15% MeOH) to afford *N*,*N*^{1}-bis-(6-chloro-2-methoxy-acridin-9-yl)-hexane-1,6-diamine **5** in 17% yield (780 mg, 2.18 mmol).

^{1}H NMR ($CDCl_3$, 360 MHz) δ ppm: 1.41 (qn, J = 6.8 Hz, 4H–2 CH_2 2), 1.69 (t, J = 6.8 Hz, 4H–2 CH_2 3), 3.63 (q, J = 6.8 Hz, 4H–2 CH_2 1), 3.91 (s, 3H O–CH_3), 4.58 (t, J = 6.8 Hz, 2H–2 NH), 7.15 (d, J = 2.5 Hz, 2H–2 CH Acr 5′), 7.28 (dd, J_1 = 1.8 Hz, J_2 = 9.4 Hz, 2H–2 CH Acr 3′), 7.40 (dd, J_1 = 2.5 Hz, J_2 = 9.4 Hz, 2H–2 CH Acr 7′), 7.96 (d, J = 9.4 Hz, 2H–2 CH Acr 4′), 7.98 (d, J = 9.4 Hz, 2H–2 CH Acr 8′), 8.05 (d, J = 1.8 Hz, 2H–2 CH Acr 1′).

^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm: 26.69 (2 CH_2), 31.73 (2 CH_2), 50.6 (2 CH_2), 99.1 (2 O–CH_3), 116.1 (2 C Acr), 123.8 (2 CH Acr), 124.4 (2 CH Acr), 124.8 (2 CH Acr), 128.5 (2 CH Acr), 131.8 (2 CH Acr), 148.4 (4 C Acr), 149.2 (2 C Acr), 149.5 (2 C Acr), 153.2 (2 C Acr), 156.1 (2 C Acr).

MS (ES) m/z: 599.2 (MH^+) (100%), 300.1 (M+2H$^+$)/2 (26.4%).

Anal. Calcd for $C_{34}H_{32}Cl_2N_4O_2{\cdot}2.5H_2O$: C, 63.35; H, 5.78; N, 8.69. Found: C, 63.82; H, 5.72; N, 8.01. MM = 644.6.

6.1.5. *N*^{1}-Acridin-9-yl-hexane-1,6-diamine (6)

A solution of 9-chloroacridine (2.49 mmol, 0.531 g), NH-Boc-hexylamine (2.81 mmol, 0.607 g) and DIEA (4.98 mmol, 822 μL) was heated at 60–80 °C for 6 h. After evaporation under reduced pressure and purification by MPLC with CH_2Cl_2/MeOH (2–10% MeOH), the amino derivative was obtained in 66% yield (1.77 mmol, 0.65 g).

This amino compound was then dissolved in TFA (20 mL) and stirred overnight at room temperature. After evaporation under reduced pressure, the crude product was passed over an ion exchange resin Dowex Cl$^-$, which afforded compound **6** after lyophilization in 60% yield (1.40 mmol, 0.548 g).

^{1}H NMR (CD_3OD, 360 MHz) δ ppm: 1.53 (m,4H–2CH_2), 1.72 (m, 2H–CH_2), 2.02 (m, 2H–CH_2), 2.95 (t, J = 7.5 Hz, 2H–CH_2), 4.14 (t, J = 7.4 Hz, 2H–CH_2), 7.52 (t, J = 7.4 Hz, 2H–2CH Acr), 7.79 (d, J = 8.1 Hz, 2H–2CH Acr), 7.90 (t, J = 7.4 Hz, 2H–CH Acr), 8.47 (d, J = 8.4 Hz, 2H–2CH Acr).

^{13}C NMR ($CDOD_3$, 50.3 MHz) δ ppm: 27.3 (CH_2), 27.6 (CH_2), 28.6 (CH_2), 30.7 (CH_2), 40.9 (CH_2), 50.5 (CH_2), 120.1 (2 C Acr), 124.0 (2 CH Acr), 125.7 (2 CH Acr), 128.9 (2 CH Acr), 132.7 (2 CH Acr), 145.5 (C Acr), 149.2 (2 C Acr).

MS (ES) m/z: 368, (100%) (MH^+).

HRMS calcd for $C_{19}H_{23}N_3$ (MH^+): 294.1970, found: 294.1958.

Anal. Calcd for $C_{19}H_{23}N_3{\cdot}2HCl{\cdot}1.5H_2O{\cdot}0.25NaCl$: C, 58.52; H, 6.72; N, 10.77. Found: C, 58.52; H, 6.85; N, 10.52. MM = 389.95.

UV–vis (H_2O): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 276 (18 400), 340 (2 470), 420 (3 930), 442 (3 310).

6.1.6. 7-(Acridin-9-ylcarbamoyl)-heptyl-ammonium chloride (7)

This compound was synthesised from 9-aminoacridine and NH-Boc-caprylic acid according to the procedure already described.[7] The crude product was then passed over ion exchange resin Dowex Cl$^-$; compound **7** was obtained in quantitative yield after lyophilisation.

^{1}H NMR (CD_3OD, 250 MHz) δ ppm: 1.40 (m, 6H–CH_2 3, 4, 5), 1.70 (m, 2H), 1.90 (m, 2H–CH_2 6), 2.80 (t, 2H–CH_2 1), 2.90 (m, 2H–CH_2 7), 7.20 (t, 1H–CH Acr), 7.50 (t, 1H–CH Acr), 7.70 (dd, 2H–2 CH Acr), 8.10 (dd, 2H–2 CH Acr), 8.30 (d, 1H–CH Acr).

^{13}C NMR (CD_3OD, 50 MHz) δ ppm: 26.3 (CH_2), 27.3 (CH_2), 28.5 (CH_2), 29.9 (CH_2), 30.1 (CH_2), 37.4 (CH_2), 40.7 (CH_2), 112.3, 119.5, 120.7, 122.7, 124.9, 125.1, 127.2, 128.5, 136.6, 138.3, 140.2, 141.5, 153.4, 175.5 (CO amide).

MS (ES) m/z: 336.3 (MH^+) (100%).

HRMS calcd for $C_{21}H_{25}N_3O$ (MH^+): 333.2076, found: 336.2079.

Anal. Calcd for $C_{21}H_{26}ClN_3O{\cdot}0.5NaCl{\cdot}2H_2O$: C, 57.70; H, 6.92; N, 9.32. Found: C, 57.71; H, 6.91; N, 9.32. MW: 437.16.

UV–vis (H_2O): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 251 (130 900), 358 (11 200), 422 (8 540).

6.1.7. Synthesis of 2-[2-(acridin-9-ylcarbamoyl)-ethylcarbamoyl]-ethyl-ammonium chloride (8)

6.1.7.1. [2-(Acridin-9-ylcarbamoyl)-ethyl]-carbamic acid *tert*-butyl ester. A mixture of Boc-β-AlaOH (4.22 mmol, 800 mg), BOP (5.07 mmol, 2.24 g) and DIEA (5.07 mmol, 840 μL) in DMF (20 mL) was stirred at room temperature for 30 min. 9-Aminoacridine hemihydrate (1.05 g, 4.22 mmol) was added dropwise to the activated ester. The reaction mixture was stirred for 24 h at 50 °C to allow the complete dissolution of the acridine. Almost all of the DMF was removed under reduced pressure and the residue was added dropwise to a stirred solution of 5% $NaHCO_3$ (100 mL). The mixture was allowed to stand for 24 h at room temperature. The resulting precipitate was filtered and dried under vacuum and [2-(acridin-9-ylcarbamoyl)-ethyl]-carbamic acid *tert*-butyl ester was obtained as an amorphous powder in 60% yield (2.52 mmol, 920 mg), after chromatography in CH_2Cl_2/MeOH (95:5).

^{1}H NMR (CD_3OD, 360 MHz) δ ppm: 1.50 (s, 9H–3 CH_3 Boc), 2.96 (m, 2H–CH_2 CO βala), 3.55 (t, J = 6.5 Hz, 2H–CH_2 NH βala), 7.64 (t, J = 8.0 Hz, 2H–2 CH Acr), 7.88 (t, J = 8.0 Hz, 2H–2 CH Acr), 8.19 (d, J = 8.0 Hz, 4H–4 CH Acr).

^{13}C NMR (CD_3OD, 50 MHz) δ ppm: 29.0 (3 CH_3 Boc), 37.5 (CH_2), 38.2 (CH_2), 80.5 (C Boc), 124.5 (CH Acr), 125.5 (CH Acr), 127.6 (CH Acr), 129.5 (C Acr), 132.2 (CH Acr), 150.4 (2 C Acr), 158.6 (CO carbamate), 174.0 (CO amide).

MS (ES) m/z: 366.2 (MH^+) (100%), 388.2 (MNa^+) (45.4%).

6.1.7.2. 2-(Acridin-9-ylcarbamoyl)ethyl-ammonium chloride. [2-(Acridin-9-ylcarbamoyl)-ethyl]-carbamic acid *tert*-butyl ester (2.52 mmol, 920 mg) was dissolved in TFA/CH_2Cl_2 (1:1) (10 mL). After stirring overnight in the dark, the solvent was evaporated in vacuo. The TFA salt **8** was obtained quantitatively and used without further purification.

6.1.7.3. {2-[2-(Acridin-9-ylcarbamoyl)-ethylcarbamoyl]-ethyl}-carbamic acid *tert*-butyl ester. A mixture of Boc-β-AlaOH (2.77 mmol, 525 mg) and CDI (2.77 mmol, 450 mg) in DMF (10 mL) was stirred at room temperature for 30 min. The TFA salt **8** and DIEA (3.78 mmol, 625 μL) were dissolved in DMF (10 mL) and added dropwise to the activated ester. The reaction mixture was stirred overnight in the dark at room temperature. DMF was removed under reduced pressure and the residue was dissolved in acetone (10 mL) and added dropwise to a stirred solution of 5% $NaHCO_3$ (100 mL). The mixture was allowed to stand for 24 h at room temperature. The resulting precipitate was filtered and

dried under vacuum. {2-[2-(acridin-9-ylcarbamoyl)-ethylcarbamoyl]-ethyl}-carbamic acid *tert*-butyl ester was obtained as a pale yellow powder in 78% yield (1.97 mmol, 860 mg).

^{1}H NMR (CD_3OD, 250 MHz) δ ppm: 1.39 (s, 9H–3 CH_3 Boc), 2.50 (t, J = 6.8 Hz, 2H–CH_2 CO βala2), 2.96 (t, J = 6.8 Hz, 2H–CH_2 CO βala1), 3.35 (t, J = 6.8 Hz, 2H–CH_2 NH βala2), 3.68 (t, J = 6.8 Hz, 2H–CH_2 NH βala1), 7.59 (t, J = 7.5 Hz, 2H–2 CH Acr), 7.83 (t, J = 7.5 Hz, 2H–2 CH Acr), 8.15 (t, J = 7.5 Hz, 4H–4 CH Acr).

^{13}C NMR ($CDCl_3$, 50 MHz) δ ppm: 28.7 (3 CH_3 Boc), 37.1 (CH_2 βala), 37.2 (CH_2 βala), 37.3 (CH_2 βala), 37.4 (CH_2 βala), 86.1 (C Boc), 123.8 (2 CH Acr + 2 C Acr), 125.5 (2 CH Acr), 126.7 (2 CH Acr), 129.0 (2 C Acr), 132.0 (2 CH Acr), 150.0 (2 C Acr), 164.8 (CO carbamate), 173.4 (CO amide), 174.1 (CO amide).

MS (ES) *m/z*: 437.3 (MH^+) (100%), 459.2 (MNa^+) (99.6%).

6.1.7.4. 2-[2-(Acridin-9-ylcarbamoyl)-ethylcarbamoyl]-ethyl-ammonium chloride (8). The previous compound (1.97 mmol, 860 mg) was dissolved in TFA/CH_2Cl_2 (1:1) (10 mL). After stirring overnight in the dark, the solvent was evaporated in vacuo. The solid was dissolved in distilled water and passed over an ion exchange resin Dowex Cl^-. The chloride salt 2-[2-(acridin-9-ylcarbamoyl)-ethylcarbamoyl]-ethyl-ammonium chloride (**8**) was obtained quantitatively, after lyophilization.

^{1}H NMR (CD_3OD, 250 MHz) δ ppm: 2.67 (t, J = 6.5 Hz, 2H–CH_2 CO βala2), 3.12 (t, J = 6.5 Hz, 2H–CH_2 CO βala1), 3.22 (t, J = 6.5 Hz, 2H–CH_2 NH βala2), 3.71 (t, J = 6.5 Hz, 2H–CH_2 CH_2 NH βala1), 7.91 (t, J = 8.0 Hz, 2H–2 CH Acr), 8.28 (q, J = 8.0 Hz, 4H–4 CH Acr), 8.15 (d, J = 8.0 Hz, 2H–2 CH Acr).

MS (ES) *m/z* : 337.2 (MH^+) (55%) 359.1 (MNa^+) (45%).

HRMS calcd for $C_{19}H_{20}N_4O_2$ (MH^+): 337.1665, found: 337.1660.

Anal. Calcd for $C_{19}H_{23}ClN_4O_2 \cdot 0.75NaCl \cdot H_2O$: C, 52.50; H, 5.33; N, 12.89. Found: C, 52.41; H, 5.39; N, 12.72. MW: 434.70.

UV–vis (H_2O): λ_{max} nm (ε mol^{-1} L cm^{-1}) = 251 (97 600), 360 (9 480).

6.1.8. pK_a determination

Spectrophotometric measurements were carried out to determine pK_a values. Solutions of acridine derivatives were prepared at a final concentration of 2.5 μM in hydrochloric acid 0.2 M containing 0.15 M NaCl. Titration were conducted with NaOH solutions (0.4 M or 0.1 M) containing 2.5 μM of acridine to avoid dilution effects and 0.15 M NaCl. Spectra were recorded between 200 and 500 nm after each addition leading to an increase of 0.5 pH unit (or 0.25 pH unit in the pK_a zone). A sigmoid curve was then obtained by plotting pH versus $A_{\lambda}AcrH/A_{\lambda Acr}$, where λAcrH and λAcr are respectively the maximum wavelengths of the protonated and the neutral species, and $A_{\lambda}AcrH$ and $A_{\lambda}Acr$ the absorbances corresponding to these specific wavelengths. The pK_a value was calculated using the following expression in KGraph 5.1 to fit the curve:

$$\frac{A_{\lambda AcrH}}{A_{\lambda Acr}} = \frac{10^{-pH}A_{\lambda AcrH}(AcrH^+) + K_aA_{\lambda AcrH}(Acr)}{10^{-pH}A_{\lambda Acr}(AcrH^+) + K_aA_{\lambda Acr}(Acr)}$$

6.2. Biological evaluation

6.2.1. *P.* falciparum strains

Both CQ-susceptible (3D7) and CQ-resistant (W2, FCR3 and Bre1) *P. falciparum* strains maintained continuously in culture were used. All strains were synchronised twice with sorbitol before use. Synchronous parasites were diluted with uninfected erythrocytes (A-positive human blood) and completed RPMI 1640 medium (Invitrogen, Paisley, United Kingdom), supplemented with 10% human serum Abcys S.A. (Paris, France) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ to achieve 0.5 parasitemia and 1.5 hematocrit. Parasites were grown under controlled atmospheric conditions that consisted of 10% O_2, 5% CO_2, and 85% N_2 at 37 °C with a humidity of 95%.

6.2.2. Measurement of in vitro antimalarial activity

Solutions of drugs were prepared in RPMI 1640 medium and distributed in triplicate into Falcon 96-well flat-bottomed plates (Becton Dickinson, Franklin Lakes, NJ) to achieve concentrations ranging from 0.006 μM to 200 μM.

For in vitro isotopic microtests, 25 μL/well of compounds and 200 μL/well of the suspension of synchronous parasitised red blood cells (final parasitemia, 0.5%; final hematocrit, 1.5%) were distributed in 96-well plates. Parasite growth was assessed by adding 1 μCi of [^{3}H]hypoxanthine with a specific activity of 14.1 Ci/mmol (Perkin–Elmer, Courtaboeuf, France) to each well. Plates were incubated for 42 hours at 37 °C in an atmosphere of 10% O_2, 5% CO_2, 85% N_2, and an humidity of 95%. Immediately after incubation the plates were frozen then thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter™ GF/B, Perkin–Elmer, Meriden, USA) and washed using a cell harvester (FilterMate™ Cell Harvester, Packard). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint™ O, Perkin–Elmer) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count™, Perkin–Elmer).

The 50% inhibitory concentration (IC_{50}), that is, the drug concentration corresponding to 50% of the uptake of [^{3}H]hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves (Riasmart™, Packard, Meriden, USA). Data were analysed after logarithmic transformation and expressed as the geometric mean IC_{50} and 95% confidence intervals (95% CI) were calculated (Stata9™, StataCorp LP, Texas, USA).

6.2.3. Cell culture and cell proliferation assay

The human cell lines KB (month epidermoid carcinoma) was obtained from ECACC (Salisbury, UK) and grown in D-MEM medium supplemented with 10% fetal calf serum (Invitrogen), in the presence of penicillin, streptomycin and fungizone in 75 cm^2 flask under 5% CO_2. Cells were plated in 96-well tissue culture microplates at a density of 650 cells/well in 200 μL medium and treated 24 h later with compounds dissolved in DMSO at concentrations ranged from 0.5 nM to 10 μM using a Biomek 3000 automate (Beckman-Coulter). Controls received the same volume of DMSO (1% final volume). After 72 h exposure MTS reagent (Promega) was added and incubated for 3 h at 37 °C: the absorbance was monitored at 490 nm and results expressed as the inhibition of cell proliferation calculated as the ratio [(OD490 treated/OD490 control) × 100]. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in separate duplicate.

6.3. Interaction with hematin

6.3.1. Reagents

Hemin chloride (H-5533), hematin (ferriprotoporphyrin IX hydroxide) (H-3281), chloroquine diphosphate (C-6628) were obtained from Sigma.

6.3.2. Microassay for β-hematin formation: BHIA

The method to assay the ability of different compounds to inhibit β-hematin formation was reported for the first time by Parapini et al.,[24] and is referred as BHIA (β-hematin inhibitory activity).

A total of 50 μL of an 8 mM solution of hemin dissolved in DMSO was introduced in eppendorfs (0.4 μmol/eppendorf); 50 μL of different compounds in DMSO or DMSO/MeOH (1:1), in doses ranging from 0 to 10 M equiv to hemin, was added to triplicate test eppendorfs. In control eppendorfs, 50 μL of water was added

(DMSO/MeOH for water-insoluble compounds). The final concentration of DMSO was kept constant at 25%. β-Hematin formation was initiated by the addition of 100 μL of 8 M acetate buffer (pH 5).

Eppendorfs were incubated at 37 °C for 18 h to allow complete reaction and were then centrifuged 4000*g* for 15 min. The soluble fraction of unprecipitated hematin was collected. The remaining pellet was resuspended with 200 μL of DMSO to remove unreacted hematin. Eppendorfs were then centrifuged again at 4000*g* for 15 min. The DMSO-soluble fraction was collected and the pellet consisting of a pure precipitate of β-hematin, was dissolved in 0.1 M NaOH for spectroscopic quantitation. An aliquot of each fraction was transferred onto a new plate and serial dilutions in 0.1 M NaOH were performed. The amount of hematin was determined by measuring the absorbance at 405 nm. The data are expressed as the concentrations of tested compounds required to inhibit haem crystallization by 50%.

6.3.3. Drug/hematin interaction assay: determination of K_d and stoichiometry

An aqueous DMSO (40%, v/v) solution of 10 μM hematin (pH 7.4) was freshly prepared by mixing 25 μL of 4 mM hematin in 0.1 M NaOH solution with 4 mL of DMSO and 1 mL of 0.02 M sodium phosphate buffer (pH 6.0) and increasing the volume up to 10 mL with double-distilled deionized water (under these conditions, hematin is monomeric).[35] For each compound, solutions containing drug and hematin combinations at the following 10 molar ratios were prepared: 0:40; 4:36; 8:32; 12:28; 16:24; 20:20; 24:16; 28:12; 32:8; 40:0. The final combined concentration of hematin plus drug in the mixtures was 10 μM. Spectra were recorded between 240 and 500 nm on a UVIKON spectrophotometer at a speed of 0.5 nm/min. Spectrophotometric data could then be analyzed by the standard equation[36] $1/\Delta A = 1/\Delta A_\infty + K_d/\Delta A_\infty \times 1/[\text{Drug}]^n$, where $\Delta A = A - A_0$, $\Delta A_\infty = A - A_\infty$ and A_0, A_∞ and A are the absorbance of the initial, final and mixed species, respectively. The linearity of the graph representing $1/\Delta A$ as a function of $1/[\text{Drug}]^n$ was then assayed with $n = 1$, $n = 2/3$, $n = 3/2\ldots$ and K_d and A_∞ could be determined graphically.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.005.

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