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The antimalarial activity of Ru–chloroquine complexes against resistant *Plasmodium falciparum* is related to lipophilicity, basicity, and heme aggregation inhibition ability near water/*n*-octanol interfaces

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Abstract

We have measured water/*n*-octanol partition coefficients, pK_a values, heme binding constants, and heme aggregation inhibition activity of a series of ruthenium– π -arene–chloroquine (CQ) complexes recently reported to be active against CQ-resistant strains of *Plasmodium falciparum*. Measurements of heme aggregation inhibition activity of the metal complexes near water/*n*-octanol interfaces qualitatively predict their superior antiparasmodial action against resistant parasites, in relation to CQ; we conclude that this modified method may be a better predictor of antimalarial potency than standard tests in aqueous acidic buffer. Some interesting tendencies emerge from our data, indicating that the antiparasmodial activity is related to a balance of effects associated with the lipophilicity, basicity, and structural details of the compounds studied.

Keywords

Chloroquine; Ruthenium; Malaria; Heme aggregation; Lipophilicity

Introduction

Malaria continues to be one of the most serious global health issues. According to the World Health Organization, 300–500 million people become infected and close to a million die of malaria each year, mostly children under 5 years of age [1]. The most important problem in the treatment of malaria is the widespread emergence of resistance to commonly used drugs, particularly chloroquine (CQ) (Fig. 1), which was the most efficacious, safe, and inexpensive alternative widely used over several decades. Modifications of the molecular structure of CQ led to other effective quinoline-based drugs such as amodiaquine and mefloquine but, unfortunately, resistance to these drugs is now also common in many parts of the world [1, 2].

Malaria parasites infect red blood cells (RBCs) of the host and degrade hemoglobin inside an acidic digestive vacuole (pH ~ 5). This process releases heme, which is lethal to the

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parasite; the toxic effects are avoided through a natural defense mechanism consisting of oxidation to ferriprotoporphyrin followed by aggregation into an insoluble innocuous malaria pigment (hemozoin) [2]; a wealth of evidence has recently led to the conclusion that hemozoin formation occurs in close association with lipids and it takes place spontaneously and rapidly at water/lipid interfaces within the digestive vacuole [3, 4]. Inhibition of hemozoin formation is the principal target of many antimalarial agents; CQ, in particular, is able to rapidly penetrate infected RBCs and accumulate within the food vacuole of the parasite, where it inhibits heme aggregation. Strong π – π molecular interactions between the planar quinoline moiety and the porphyrin ring of monomeric heme units are often associated with inhibition of hemozoin formation [2, 5]. An alternative proposal invokes stereospecific binding of the drug to certain hemozoin crystal faces as the basis for inhibition of crystal growth [6].

Although the precise mechanism of CQ resistance is not yet fully understood, it has been often associated with polymorphisms within the *pfcr*t gene, which encodes a 48.6-kDa protein PfCRT localized at the membrane of the food vacuole, believed to function as a transmembrane transporter (or channel) [2, 7–9]. Mutations in this protein are linked to CQ resistance, particularly K76T, which involves the change of a charged amino acid, lysine, to uncharged threonine; mutated PfCRT is able to bind and expel CQ from the digestive vacuole. It further appears that because of the specific mutation involved, the ability of compounds to be extruded through PfCRT decreases with increasing lipophilicity [9–11]; this is likely related to the fact that hemozoin crystals appear to grow from water/lipid interfaces into lipid nanospheres within the food vacuole [3, 4] and therefore increasingly lipophilic drugs would accumulate better near water/lipid interfaces and should consequently be more efficient at inhibiting aggregation.

Because of resistance to CQ, first-line antimalarial treatments today are based on artemisinin and its derivatives, which are highly active and fast acting, particularly against *Plasmodium falciparum*. These drugs act by a different and highly controversial mechanism, possibly involving the Fe(II)-mediated homolysis of the endoperoxide moiety to generate free radicals capable of forming covalent adducts with parasite components [1, 2]. Malaria parasites have not yet developed any significant resistance to this family of drugs, but the short plasma half-lives of endoperoxide drugs also result in parasite recrudescence when used as the sole therapy. In consequence, the World Health Organization currently recommends a combination therapy (artemisinin plus amodiaquine, lumefantrine, or mefloquine), which provides an effective treatment. Nevertheless, this method poses certain challenges related to reaching the required concentrations of both drugs in the blood at the right time, owing to different solubility and pharmacokinetics characteristics of the individual components [12].

A different approach toward new antimalarial chemotherapies capable of overcoming resistance involves metal-based drugs containing the CQ moiety. In early work we demonstrated that coordination of CQ to metal-containing fragments (Ru [13, 14], see the example in Fig. 2, Rh [13], Au [15, 16]) leads to enhanced activity against CQ-resistant strains of *P. falciparum*; Navarro et al. [17] reported related Ir–CQ derivatives that are active in vivo against rodent malaria. Biot, Brocard, and coworkers were able to integrate a ferrocenyl unit into the side chain of a CQ-like structure through covalent linking to one of the cyclopentadienyl units of the organometallic fragment. This led to the compound ferroquine (Fig. 2), which is highly active and selective against CQ-resistant *P. falciparum* and is currently in clinical development; the ruthenium analogue was also prepared and it displayed very similar biological activity [18, 19]. The groups of Moss and Chibale [20–22] have reported a number of other iron- and ruthenium-containing ferroquine-like derivatives with different substituents in the aliphatic chain and found them to be active against CQ-

sensitive and CQ-resistant malaria parasites, with small differences between iron and ruthenium compounds. Although the mechanisms of biological action have not been studied in many cases, metal-based antimalarial agents derived from CQ appear to maintain heme aggregation as the main target.

Of particular relevance to this work, we showed that the complex $[\text{RuCl}_2(\text{CQ})]_2$ displayed enhanced activity in vitro against CQ-resistant FCB1 and FCB2 strains of *P. falciparum* and in vivo against *P. berghei* (rodent malaria) [13]; we also investigated the mechanism of action of this complex and concluded that the role of the metal-containing fragment is an alteration of the structure, the basicity, and the lipophilicity of CQ to make it less recognizable to the parasite's defense mechanism [14]. We also recently reported a second generation of Ru–CQ complexes that involve a more elaborate molecular design containing π -bonded arene and other ancillary ligands (Fig. 3); these complexes display antimalarial activity, particularly against CQ-resistant strains of *P. falciparum*, as well as antitumor activity against colon cancer and liposarcoma cell lines [23].

In this paper, we describe some physicochemical features of these new Ru–CQ derivatives, namely, lipophilicity, basicity, interaction with hematin, and heme aggregation inhibition activity (HAIA) and discuss possible relations with their antiplasmodial activity.

Materials and methods

General

All complexes were synthesized as previously described [23]. Hemin, buffers, and solvents were purchased from Sigma-Aldrich. Solvents were purified through a PureSolv purification unit from Innovative Technology; all other chemicals were used as received. Spectrophotometric studies were performed with an Agilent 8453 diode-array spectrophotometer equipped with an HP 89090 Peltier temperature controller. pH measurements were performed using an Oakton pH/CON 510 instrument.

Interaction with hematin

The binding constants for binding of hematin with chloroquine diphosphate (CQDP) and all the ruthenium complexes were determined by spectrophotometric titration according to literature methods [24, 25] adapted as previously described by us [14]. In short, hemin (3.5 mg) was dissolved in dimethyl sulfoxide (DMSO) (15 ml) to obtain a stock solution. A 4 μM working solution of hemin was freshly prepared by diluting the stock solution in a buffer consisting of 40% v/v DMSO and 20 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 4.97). This mixture was titrated with a 1.03 mM solution of each drug in the same buffer at 25 °C by monitoring the absorbance of the Soret band (402 nm). Blank measurements were performed at the same wavelength by titration of 40% v/v buffer with the solution of each complex to correct for the absorbance of the drugs. The data were fitted to the equation $A = (A_0 + A_\infty K[C]) / (1 + K[C])$ for a 1:1 complexation model using nonlinear least squares fitting, strictly following the procedure of Egan et al. [24] with omission of the first few data points at very low drug-to-hematin ratio. A_0 is the absorbance of hemin before addition of the drug, A_∞ is the absorbance for the drug–hemin adduct at saturation, A is the absorbance at each point of the titration, and K is the conditional association constant.

HAIA in acidic buffer

The ability of each complex to inhibit heme aggregation was quantified using a modification of the method reported by Parapini et al. [26] as described in a previous paper [14]. In short, triplicate samples at different drug-to-hemin molar ratios, containing 1 ml of an 8 mM solution of hemin in DMSO, 1 ml of drug in deionized water (or 20% DMSO/80% water for

less soluble complexes), and 2 ml of 8 M acetate buffer (pH 4.9) were prepared (solutions of each compound in water were of appropriate concentrations to achieve drug-to-hemin molar ratios in the range 0–2). The mixtures were incubated for 24 h at 37 °C to allow for complete reaction and then centrifuged for 20 min. The soluble fraction was separated from each sample and the remaining β -hematin pellet was washed with 4 ml of DMSO, centrifuged again, and finally the pellet of pure β -hematin was dissolved in NaOH (0.1 M) for spectrophotometric quantification. Control experiments using no drug or CQDP were carried out under analogous conditions.

HAIA at a water/*n*-octanol interface

Inhibition of the heme aggregation near the interface of aqueous buffer/*n*-octanol mixtures was studied following the method described by Egan et al. [27] with modifications as previously described [14]. To establish a baseline for the aggregation process, hemin was dissolved in 0.1 M NaOH solution to generate hematin and acetone was added until the acetone-to-water ratio was 4:6; the final solution contained 15 mg hematin/ml. A sample of this solution (200 μ l) was carefully introduced close to the interface between *n*-octanol (2 ml) and aqueous acetate buffer (8 M; 5 ml pH 4.9) in a cylindrical vial with an internal diameter of 2.5 cm. The mixture was incubated at 37 °C for 60 min and at the end of the incubation period it was stirred to ensure the transfer of all solid particles to the aqueous layer. The product (β -hematin) was isolated by centrifugation. The pellet was collected and washed with DMSO (4 ml), centrifuged again for 20 min, washed with 2 ml of ethanol, and finally dissolved in 25 ml of 0.1 M NaOH for spectrophotometric quantification at 386 nm. For the aggregation inhibition activity measurements, the appropriate amount of each drug (23 mM in water) to yield drug-to-hemin concentration ratios in the range 1–6 was added to the acetate buffer/*n*-octanol mixture; after stirring for 30 min to equilibrate the drug between the two phases, the hematin solution was added close to the interface and the procedure was followed as described above. All experiments were performed in triplicate.

Determination of partition coefficients and pK_a values

The distribution of all complexes between *n*-octanol and water was studied by use of the stir-flask method [28–30]. A mixture of 10 ml *n*-octanol and 10 ml of water (each saturated in the other) was stirred for 30 min at the desired temperature after the right amount of the sample to be analyzed (approximately 0.7 mmol) had been added. The pH was adjusted to the desired value by addition of a 0.1 M solution of H_3PO_4 (10–20 μ l). Once the equilibrium had been reached, the organic and aqueous phases were separated and centrifuged. Finally, the concentration of drug in each phase was measured spectrophotometrically to determine values of $D = [\text{drug}] \text{ (in octanol)} / [\text{drug}] \text{ (in water)}$. Experiments were carried out in triplicate.

For the determination of pK_a values, a 1 mM solution of each compound in deionized water was titrated by following pH changes at room temperature (23.0 ± 2.0 °C) using 0.03 M NaOH (10- μ l additions). Titration plots were generated and the pK_a values were obtained from the maxima of the first derivative plots (dpH/dV) versus V , where V represents the volume of the titrant added.

Results

Interaction of complexes 1–4 with hematin

Since our most likely target of biological action is the heme aggregation process, as a first step we studied the interaction of complexes **1–4** with hematin by spectrophotometric titration at the 402-nm Soret band in aqueous DMSO at pH 4.97, which simulates the conditions within the acidic food vacuole of the parasite; in separate experiments, the

stability of the complexes at low pH was verified spectrophotometrically. An example of the spectroscopic measurements is shown for complex **1** in Fig. S1. The data were fitted to a 1:1 association model (Fig. S1, insert) [24, 25] to yield the values of $\log K$ collected in Table 1.

The $\log K$ value of 4.82 ± 0.02 obtained for CQDP under our experimental conditions (pH 4.97) is in excellent agreement with the value of 4.8 previously reported by Egan et al. [24] at pH 5.6. A similar association constant was reported by Biot et al. [25] for ferroquine ($\log K = 4.95 \pm 0.05$) at pH 7.5. More important for our discussion is the fact that the values for the association constants of the Ru–CQ complexes are only slightly lower than the value for CQDP under our experimental conditions and are very similar to each other, indicating that the metal–CQ derivatives **1–4** interact with hematin in a similar way and to a comparable extent as CQDP under these conditions.

HAIA of complexes **1–4** in acetate buffer and at an acetate buffer/*n*-octanol interface

A number of HAIA assays have been discussed in the literature [3, 4]. By using a variation of the β -hematin inhibition activity method reported by Parapini et al. [26], we measured the ability of CQDP and of complexes **1–4** to inhibit the formation of β -hematin after 24 h under strictly comparable conditions, starting from commercial hemin in acetate buffer at pH 4.9. The data collected in Table 2 (center column) indicate that all the Ru–CQ compounds are able to inhibit the heme aggregation process; furthermore, while complex **1** appears as somewhat less active than CQDP in this assay, complexes **2–4** display an activity very similar to that of CQDP and to each other.

More interestingly, when the heme aggregation inhibition assay was conducted in an aqueous/*n*-octanol mixture at pH 4.9, following an adaptation of the procedure described by Egan et al. [27] in which the hematin is carefully introduced close to the interface after the drug has been equilibrated between the two phases, the overall aggregation process is much faster (60 min) and the activity trend changes drastically with respect to the results of the assay in aqueous buffer.

As shown in Table 2 (right column), all complexes are significantly more potent than CQDP for inhibiting heme aggregation near the interface. Complex **1**, which appeared as the least active in aqueous buffer, displays the highest activity at the interface, twice as active as CQDP; although no significant differences were observed in the activity of complexes **2–4** in the experiments performed in an aqueous medium ($2 \sim 3 \sim 4 \sim \text{CQDP} > 1$), the values measured at the interface for the four compounds follow a clear trend: $1 > 3 > 4 > 2 > \text{CQDP}$.

Partition coefficient and pK_a measurements

To probe the influence of the lipophilicity of each complex on the heme aggregation and antimalarial activity of the Ru–CQ complexes, partition (or distribution) coefficients were measured in water/*n*-octanol mixtures at two pH values (4.9 and 6.6) that can be related to vacuolar and cytosolic conditions, respectively. The corresponding $D(\text{pH})$ values are collected in Table 3.

We note that at the vacuolar pH (4.9) the Ru–CQ complexes are generally more lipophilic than CQDP, with the exception of complex **4**, which is highly hydrophilic. The general lipophilicity trend that can be extracted from the data at pH 4.9 is $1 > 3 > 2 > \text{CQDP} \gg 4$. On the other hand, at pH 6.6 only complex **4** accumulates preferentially in the aqueous phase ($D < 1$), while the other Ru–CQ complexes are less lipophilic than CQDP; the lipophilicity trend in this case is $\text{CQDP} > 3 > 1 > 2 \gg 4$.

Concerning basicity measurements, we obtained values of pK_{a1} and pK_{a2} of 9.69 and 8.12 for CQDP, which are similar to previously reported values [25, 31]. For the metal complexes, we first note that in the π -bonded Ru–CQDP complex **4** the basicity is modestly reduced with respect to free CQDP (pK_{a1} and pK_{a2} of 9.31 and 7.91). On the other hand, in complexes **1–3**, the quinoline nitrogen is blocked by coordination to ruthenium and as a consequence only pK_{a2} values are measured, and they indicate a lowered basicity with respect to pK_{a1} of CQDP, following the trend **1** < **2** > **3**. In the commonly accepted mechanism of action of CQ, the molecule is thought to travel in its neutral form until it enters the acidic food vacuole, where it is protonated and thereby trapped. Thus, it is interesting to compare the extent to which new drugs are able to accumulate within the food vacuole, in comparison with CQ. Vacuolar accumulation ratios (VAR) were calculated according to $VAR = 10^{[\log D(6.6) - \log D(4.9)]}$, as previously reported by Biot et al. [31]; the corresponding values are included in Table 3.

Discussion

The antimalarial potential of metal–CQ complexes against resistant strains of *P. falciparum* has been demonstrated in a number of cases and it is generally accepted that these compounds preserve the heme aggregation process as the main target of biological action [13–22]. Complexation to a metal results in a modification of the CQ molecule that is sufficiently drastic to hamper the action of the structure-specific PfCRT protein responsible for excreting CQ from the food vacuole. The enhanced activity observed for metal–CQ derivatives may thus be related in the first place to a higher accumulation of the drug within the food vacuole of resistant parasites. An example of such a structural modification is the complex $[RuCl_2(CQ)]_2$, which we found to be about 5 and 2.5 times more active than CQ against resistant FCB1 and FCB2 strains of *P. falciparum* [13]. We noted that this complex is more lipophilic than CQ at pH 4.9 and that the enhanced antiparasmodial activity correlates with the HAIA only if the measurements are carried out at a water/*n*-octanol interface [14]. These findings are in line with recent work indicating that heme aggregation takes place preferentially at water/lipid interfaces [3, 4] and with earlier reports relating increased lipophilicity of drugs with a lower tendency for them to be excreted from the food vacuole of resistant parasites [9–11]. We reasoned that if a given drug is less prone to be expelled by PfCRT and it accumulates near water/lipid interfaces, it should display a higher HAIA, which translates into a higher antimalarial potency. Consequently, we suggested that HAIA measurements at water/*n*-octanol or water/lipid interfaces could be better predictors of the antimalarial potential of a drug than commonly employed assays in acidic buffer [14]. To provide further support for these concepts and explore their general applicability, we now relate the present study on the HAIA and other physicochemical features of complexes **1–4** with their antiparasmodial activity recently reported elsewhere against several strains of CQ-sensitive and CQ-resistant *P. falciparum* [23].

Lipophilicity and basicity versus HAIA

First we note that drug lipophilicity, as measured by water/*n*-octanol partition coefficients (*D*), does not display any correlation with HAIA measured in aqueous buffer (Fig. 4a); interestingly, the data for CQ and the Ru–CQ complexes in Fig. 4b, measured near the aqueous/*n*-octanol interface, clearly show that the HAIA of complexes **1–3** increases smoothly as the lipophilicity increases; this is reasonable, since the effective concentration of increasingly lipophilic compounds will be higher near the interface, where the aggregation process is taking place. Complexes **1–3** display a considerably lower basicity than CQ owing to the fact that the most basic site, the quinoline nitrogen atom, is blocked by the metal and cannot be protonated. Nevertheless, their HAIA is consistently higher than that of CQDP at the interface; this implies that at a water/*n*-octanol interface at pH 4.9,

lipophilicity is a dominant factor over basicity in determining HAIA. Complex **4**, on the other hand, does not fall within the correlation in Fig. 4b; although this compound is much less lipophilic and only moderately less basic than CQ (Table 3) it displays a higher inhibitory power. Undoubtedly, other factors besides lipophilicity and basicity must be intervening to produce an inhibitory activity of complex **4** at the interface superior to that of free CQ. The lack of correlation observed in this case is most likely related to the fact that the structure of **4**, containing a diprotonated π -bonded CQDP ligand and a high overall charge of +4 (Fig. 3), is very different from the structures of all the other complexes under study, which are monocationic or dicationic and contain neutral CQ bonded to ruthenium through the nitrogen atom of the quinoline ring.

HAIA versus antiparasmodial activity

It is more interesting to compare the HAIA values in Table 2 with the antimalarial potency of the Ru–CQ compounds against CQ-sensitive and CQ-resistant strains of *P. falciparum* summarized in Table 4 [14]. The HAIA values measured in aqueous buffer predict complexes **2–4** to be of activity similar to that of CQ and complex **1** to be less active; in contrast, the results of experiments carried out at water/*n*-octanol interfaces predict all complexes to be more active than CQ.

The IC₅₀ values measured for the activities of complexes **1–4** against the CQ-resistant strains Dd2, W2, and K1 (Table 4) demonstrate that the metal complexes are indeed consistently more active than CQ, in qualitative agreement with predictions made on the basis of HAIA measurements near water/*n*-octanol interfaces. On the other hand, complexes **1–4** are generally less active than CQ against the CQ-sensitive FCB1, PFB, and F32 strains of *P. falciparum*; for 3D7 parasites, also CQ-sensitive, compounds **1–4** are all about twice as potent as CQ. We cannot at this point offer an explanation for the difference in behavior of 3D7 with respect to other CQ-sensitive strains.

Nevertheless, an important conclusion from this set of data is that measuring HAIA at water/*n*-octanol interfaces may be in general a better predictor of antimalarial potential against CQ-resistant parasites than assays performed in acidic buffer.

Lipophilicity and basicity versus antiparasmodial activity

We have shown that lipophilicity trends of structurally related compounds correlate well with HAIA values measured at aqueous/*n*-octanol interfaces; also, good qualitative correlations were observed between lipophilicity and antiparasmodial activities. It is interesting to consider other possible factors affecting antimalarial activity in more detail. Careful examination of the IC₅₀ versus lipophilicity data in Fig. 5 points to an additional interesting trend in the activities of the structurally related subset of complexes **1–3** against CQ-resistant parasites. In all cases, the antiparasmodial activities are higher than that of CQDP, presumably owing to the enhanced lipophilicity of the metal complexes. Although the activities do not correlate linearly with lipophilicity, they consistently follow the order **3** > **2** > **1**, going through a maximum corresponding to complex **3** in our series.

The fact that such maxima are not predicted by our HAIA data measured at the aqueous (pH 4.9)/*n*-octanol interface reflects the much higher complexity of the antimalarial test, as compared with the model HAIA assay; in an antimalarial test performed on infected RBCs the drug needs to cross three membranes (of the RBC, the parasite, and the digestive vacuole) to reach the target, and any considerations related to drug transit through this system are essentially ignored in the chemical assays, which simply model the inside of the food vacuole. One important factor to consider is that for it to accumulate in the food vacuole, the drug must travel down a pH gradient from approximately 7.5 to approximately

4.5 and, therefore, the basicity of each complex becomes an important additional feature to consider. Complexes **1–3** are all less basic than CQDP and therefore, in the absence of other effects, they would all be expected to accumulate less in the food vacuole, as predicted by the VAR values collected in Table 3. We believe that in the case of CQ-resistant parasites, the dominant factors for the complexes to reach and remain in the food vacuole are their modified molecular structures and their enhanced lipophilicity with respect to free CQ. This explains the higher activity of the complexes in relation with CQ, which would be more effectively expelled from the food vacuole by PfCRT. Nevertheless, once the Ru–CQ combinations overcome the resistance mechanism, an influence of the relative basicity of the different complexes on vacuolar drug accumulation may also be expected. In this respect, a higher VAR value indicates a greater tendency of a drug to accumulate within the acidic food vacuole, but it says nothing about the tendency for it to concentrate near the water/lipid interfaces, which is dominated by the drug lipophilicity. The basicity differences observed between complexes **1–3** could nevertheless account for the deviations from linearity observed in the correlation trends between lipophilicity and antimalarial activity. Indeed, the activity values observed in Fig. 5 follow the trend **3** > **2** > **1**, consistent with the basicity tendencies (pK_a values **3** > **2** > **1**; VAR **3** > **2** $\approx$ **1**).

We thus conclude that the overall potency of our π -arene–Ru–CQ complexes against resistant malaria parasites results from an important structural modification of the CQ molecule, coupled with a major lipophilicity effect and a more subtle influence of the basicity. This is in excellent agreement with our previous mechanistic proposal for the complex $[\text{RuCl}_2(\text{CQ})]_2$, in which the activity against resistant malaria was ascribed to an alteration of the structure, the basicity, and the lipophilicity of CQ by the presence of the metal [14]. Very recently, Biot et al. [31] reevaluated the mechanism of action of ferroquine through the study of the antimalarial activity of N4-substituted ferroquine derivatives in relation to their basicity and lipophilicity. For ferroquines, both the vacuolar accumulation and the lipophilicity at pH $\sim$ 5 are consistently higher than those of CQ. The authors suggest that the high antimalarial activity of ferroquine against resistant parasites is a combination of transport phenomena, localization of the drug near water/lipid interfaces, and heme aggregation inhibition.

Concerning the CQ-sensitive strains of *P. falciparum* FCB1, PFB, and F32, the activities of the Ru–CQ complexes are generally lower than those of CQ, in agreement with the lower basicity and lower VAR values of the complexes, as compared with CQ, when resistance mechanisms are not operative. A curious inverse correlation between the lipophilicity of the complexes and their antimalarial activity is observed (Fig. 6); the activity of complexes **1–3** actually decreases with increasing lipophilicity. No correlations could be established in the case of the 3D7 strain, or for complex **4**.

Conclusion

We have measured the water/*n*-octanol partition coefficients, pK_a values, heme binding constants, and HAIA in water and at water/*n*-octanol interfaces of a series of ruthenium– π -arene–CQ complexes recently reported to be active against CQ-resistant strains of *P. falciparum*. Some interesting tendencies emerge from our data indicating that the antiparasmodial activity is related to a delicate balance of effects related to the lipophilicity, the basicity, and structural details of the compounds studied, where the lipophilicity and the structural features appear to play the most prominent roles. We further found that measuring HAIA of potential drugs near water/*n*-octanol interfaces may be a better predictor of antimalarial potency than the standard tests in aqueous acidic buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Chloroquine (CQ)

Ferroquine

Fig. 2.
Examples of metal-based derivatives of CQ with antimalarial properties

Fig. 3.
Solution structures of Ru-(η^6 -arene)CQ complexes with antimalarial activity [14]

Fig. 4. Correlation between partition coefficients and heme aggregation inhibition activity for chloroquine diphosphate and complexes **1–3** **a** in aqueous acetate buffer at pH 4.9 and **b** at an aqueous phosphate buffer (pH 4.9)/*n*-octanol interface

Fig. 5. Correlation between lipophilicity and antiplasmodial activity against CQ-resistant strains of *Plasmodium falciparum* Dd2 (*left*), K1 (*center*), and W2 (*right*) for complexes 1–3 (values for D from Table 4; values for IC_{50} from [14])

Fig. 6. Correlation between lipophilicity and antiplasmodial activity against CQ-sensitive strains of *P. falciparum* FCB1 (left), PFB (center), and F32 (right) for complexes 1–3 (values for D from Table 4; values for IC_{50} from [14])

Table 1

Binding constants for binding to hemin at pH 4.97 from spectrophotometric titration experiments (for details see “Materials and methods”)

Compound	log <i>K</i>
CQDP	4.82 ± 0.02
[(<i>p</i> -Cymene)Ru ^{II} Cl ₂ (CQ)] (1)	4.57 ± 0.08
[(Benzene)Ru ^{II} Cl ₂ (CQ)] (2)	4.67 ± 0.03
[(<i>p</i> -Cymene)Ru ^{II} (H ₂ O) ₂ (CQ)][BF ₄] ₂ (3)	4.52 ± 0.16
[(<i>p</i> -Cymene)Ru ^{II} (π -CQDP)][BF ₄] ₂ (4)	4.71 ± 0.03

CQ chloroquine, *CQDP* chloroquine diphosphate

Table 2

Heme aggregation inhibition activity in acetate buffer (pH 4.9) and at an acetate buffer (pH 4.9)/*n*-octanol interface (for details see “Materials and methods”)

Compound	IC ₅₀ in aqueous buffer ^a	IC ₅₀ at interface ^b
CQDP	0.69 (1.0)	2.92 (1.0)
[(<i>p</i> -Cymene)Ru ^{II} Cl ₂ (CQ)] (1)	0.96 (0.7)	1.47 (2.0)
[(Benzene)Ru ^{II} Cl ₂ (CQ)] (2)	0.67 (1.0)	2.42 (1.2)
[(<i>p</i> -Cymene)Ru ^{II} (H ₂ O) ₂ (CQ)][BF ₄] ₂ (3)	0.65 (1.1)	1.73 (1.7)
[(<i>p</i> -Cymene)Ru ^{II} (π -CQDP)][BF ₄] ₂ (4)	0.67 (1.0)	2.15 (1.4)

IC₅₀ is the drug-to-hemin ratio required to inhibit 50% of heme aggregation against a control experiment in the absence of drugs. Values in *parentheses* are the relative activity with respect to CQDP.

^aAfter 24-h reaction

^bAfter 1-h reaction

Table 3

Partition coefficients and pK_a values of complexes **1–4** (for details see “Materials and methods.”)

Compound	$D(4,9)$	$D(6,6)$	pK_{a1}	pK_{a2}	VAR
CQDP	0.15 ± 0.01	6.61 ± 0.64	9.69	8.12	44
$[(p\text{-Cymene})Ru^II Cl_2(CQ)]$ (1)	0.41 ± 0.08	3.29 ± 0.34	–	7.91	8
$[(\text{Benzene})Ru^II Cl_2(CQ)]$ (2)	0.19 ± 0.02	1.27 ± 0.06	–	7.99	7
$[(p\text{-Cymene})Ru^II(H_2O)_2(CQ)][BF_4]_2$ (3)	0.31 ± 0.01	4.76 ± 0.06	–	8.92	15
$[(p\text{-Cymene})Ru^II(\pi\text{-CQDP})][BF_4]_2$ (4)	0.038 ± 0.008	0.66 ± 0.02	9.31	7.91	17

$D(pH) = [\text{compound}]$ in octanol/[compound] in buffer at each pH value
 VAR vacuolar accumulation ratio

Table 4

Antimalarial activity of Ru–CQ complexes against *Plasmodium falciparum* (data from [23])

Strain	IC ₅₀ (nM)	CQDP			
		1	2	3	4
FcB1	45.6 ± 4.5 (1.0)	120.0 ± 4.0 (0.3)	81.6 ± 1.2 (0.5)	96.5 ± 6.0 (0.4)	65–70 (0.6)
PFB	58.3 ± 8.5 (1.0)	109.0 ± 15.1 (0.5)	79.0 ± 4.9 (0.7)	122.6 ± 15.2 (0.5)	–
F32	8.2 ± 1.3	11.5 ± 1.8 (0.7)	13.2 ± 0.2 (0.6)	22.5 ± 0.4 (0.4)	–
3D7	39.5 ± 7.0	19.6 ± 3.4 (2.0)	17.9 ± 1.5 (2.2)	19.5 ± 2.1 (2.0)	19.3 ± 2.8 (2.0)
Dd2	1,184 ± 188	483 ± 110 (2.5)	442 ± 30 (2.7)	234 ± 41 (5.0)	557 ± 49 (2.1)
K1	1,883 ± 165	600 ± 87 (3.1)	508 ± 84 (3.7)	353 ± 61 (5.3)	529 ± 97 (3.6)
W2	2155	1,667 (1.3)	1,619 (1.3)	906 (2.4)	2,549 (0.8)

Values in *parentheses* are relative activity. Relative activity = IC₅₀(CQDP)/IC₅₀(complex)