Published in final edited form as:

J Biol Inorg Chem. 2008 June ; 13(5): 703–712.

The mechanism of antimalarial action of the ruthenium (II)-chloroquine complex $[\text{RuCl}_2(\text{CQ})]_2$

Alberto Martínez, Chandima S. K. Rajapakse, Becky Naoulou, Yasemin Kopkalli, Lesley Davenport, and Roberto A. Sánchez-Delgado

Chemistry Department, Brooklyn College and The Graduate Center, The City University of New York, 2900 Bedford Avenue, Brooklyn, NY 11210, USA

Abstract

The mechanism of antimalarial action of the ruthenium-chloroquine complex $[\text{RuCl}_2(\text{CQ})]_2$ (**1**), previously shown by us to be active in vitro against CQ-resistant strains of *Plasmodium falciparum* and in vivo against *P. berghei*, has been investigated. The complex is rapidly hydrolyzed in aqueous solution to $[\text{RuCl}(\text{OH}_2)_3(\text{CQ})]_2 [\text{Cl}]_2$, which is probably the active species. This compound binds to hematin in solution and inhibits aggregation to β -hematin at pH ~ 5 to a slightly lower extent than chloroquine diphosphate; more importantly, the heme aggregation inhibition activity of complex **1** is significantly higher than that of CQ when measured at the interface of *n*-octanol-aqueous acetate buffer mixtures under acidic conditions modeling the food vacuole of the parasite. Partition coefficient measurements confirmed that complex **1** is considerably more lipophilic than CQ in *n*-octanol-water mixtures at pH ~ 5 . This suggests that the principal target of complex **1** is the heme aggregation process, which has recently been reported to be fast and spontaneous at or near water-lipid interfaces. The enhanced antimalarial activity of complex **1** is thus probably due to a higher effective concentration of the drug at or near the interface compared with that of CQ, which accumulates strongly in the aqueous regions of the vacuole under those conditions. Furthermore, the activity of complex **1** against CQ-resistant strains of *P. falciparum* is probably related to its greater lipophilicity, in line with previous reports indicating a lowered ability of the mutated transmembrane transporter PfCRT to promote the efflux of highly lipophilic drugs. The metal complex also interacts with DNA by intercalation, to a comparable extent and in a similar manner to uncomplexed CQ and therefore DNA binding does not appear to be an important part of the mechanism of antimalarial action in this case.

Keywords

Malaria; Chloroquine; Ruthenium; Heme aggregation; Lipophilicity

Introduction

Malaria continues to be one of the major infectious disease killers throughout the tropical regions of the planet, despite being preventable and curable. Over 500 million people become severely ill and more than one million die every year, mainly in Africa; about 40% of the world's population is at risk of contracting malaria, particularly in the poorest countries. Four different parasites of the *Plasmodium* genus affect humans—*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*; the first two are the most common and *P. falciparum* causes the most deadly form of the disease [1,2]. Since the discovery and purification of quinine from the bark of the cinchona tree, several antimalarial drugs have become available, notably quinolines

[chloroquine (CQ), amodiaquine, mefloquine, primaquine], inhibitors of enzymes required for folate metabolism (pyrimethamine/sulfadoxine, proguanil), and endoperoxides derived from the natural product artemisinin [3-6]. CQ (Fig. 1) has been the most successful and widely used antimalarial drug for several decades but its efficacy is now severely compromised by the emergence of resistant strains, particularly of *P. falciparum*. This translates into an urgent need for the development of novel efficacious antimalarials, which in turn requires a deep understanding of the mechanisms of action of these drugs at the molecular level [4,7].

Malaria parasites infect red blood cells of the host and degrade hemoglobin inside an acidic vacuole (pH ~ 5). This process releases heme, which is toxic to the parasite but it is handled through a natural defense mechanism consisting of oxidation to ferriprotoporphyrin [Fe(III) PPIX] followed by aggregation into an insoluble inert malaria pigment (hemozoin). A wealth of evidence indicates that the main mode of action of CQ and other quinoline-based drugs is the inhibition of heme aggregation and hemozoin formation [8-10]. Although the structure of hemozoin has been solved and shown to be identical to that of synthetic β -hematin [11], the details of the aggregation process are not well understood. There is now general agreement on the predominance of the heme aggregation inhibition mechanism for quinoline-based drugs, but other important effects that have been noted in relation to antimalarial activity include lipophilicity and changes in basicity. DNA binding was considered the principal mechanism of action of CQ in the early literature [12] but this hypothesis has been largely abandoned even though CQ is known to bind to DNA and acridine-based antimalarial drugs are believed to act predominantly or partly by DNA binding mechanisms [13-15]. The matter is further complicated by the incomplete picture available for the mechanism of resistance to CQ [16-19]; this lack of detailed knowledge limits the possibilities of rationally redesigning this important drug in order to circumvent resistance. Nevertheless, the need for novel chemotherapeutic approaches, together with the historical success of CQ, makes it worthwhile to continue attempting to develop new CQ-derived active compounds.

The great success of platinum-based and other metal-based drugs in the chemotherapy of cancer has stimulated recent developments in inorganic medicinal chemistry for applications other than treatment of cancer [20]. We have previously discussed the concept of enhancing the activity of organic compounds of known therapeutic value by coordination to a metal-containing fragment through a metal-drug synergy [21,22]. We demonstrated the merit of this approach against *Trypanosoma cruzi*, the causative agent of Chagas disease, using metal complexes of clotrimazole and ketoconazole [23-26]; some of those complexes were also found to be active as antitumor agents [27]. We also modified CQ through coordination to metal-containing fragments, notably ruthenium [28], rhodium [28], and gold [29,30], and found enhanced antimalarial activity in several instances. Ferroquine, a derivative of CQ containing a ferrocenyl group in the side chain of the molecule, has proved to be highly efficient and selective against CQ-resistant malaria parasites [31,32]. Of particular interest to the present work, we previously reported that the complex $[\text{RuCl}_2(\text{CQ})]_2$ (**1**; Structure 1) is considerably more active than chloroquine diphosphate (CQDP) in in vitro tests against two CQ-resistant strains of *P. falciparum* (FCB1 and FCB2) and in vivo against *P. berghei*; in the latter case no signs of acute toxicity were observed in test animals after prolonged treatment [28]. In view of these promising results, we undertook an investigation of the possible mechanism of action of this novel metal-CQ complex, the results of which are reported in this paper.

Materials and methods

General

$[\text{RuCl}_2\text{CQ}]_2$ (**1**) was synthesized as previously described [28]. Calf thymus (CT) DNA, hemin, buffers, and solvents were purchased from Sigma-Aldrich. Solvents were purified by use of a PureSolv purification unit from Innovative Technology; all other chemicals were used as

received. Spectrophotometric studies and thermal denaturation experiments were performed using an Agilent 8453 diode-array spectrophotometer equipped with an HP 89090 Peltier temperature-control accessory. Steady-state fluorescence measurements were carried out using a Spex Fluorolog Tau 2 fluorimeter (SPEX-Horiba Instruments, NJ, USA) equipped with a thermostatted cuvette holder.

Interaction of complex 1 with hematin

The binding constants of CQDP and of complex 1 with hematin were determined by spectrophotometric titration according to the method described by Egan et al. [33,34]. In short, hemin (3.5 mg) was dissolved in 15 mL of dimethyl sulfoxide (DMSO) to obtain a stock solution. A 4 μ M working solution of hemin was freshly prepared by diluting the stock solution in a buffer consisting of 40% v/v of DMSO and 20 mM tris(hydroxymethyl)amino-methane hydrochloride (Tris-HCl; pH 7.4). This mixture was titrated with a 1.03 mM solution of complex 1 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 CQDP or complex 1 in order to correct for the absorbance of the drugs. The process was then repeated in 40% v/v DMSO and 20 mM 2-morpholinoethanesulfonic acid buffer (pH 4.97). Finally 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. with omission of the first few data points at very low drug-to-hematin ratio, where the fit is poor. A_0 is the absorbance of hemin before addition of CQDP or complex 1, 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.

Heme aggregation inhibition in acidic buffer

The ability of complex 1 to inhibit heme aggregation was quantified using a variation of the β -hematin inhibition activity method reported by Parapini et al. [35]. Triplicate samples at different drug-to-hemin molar ratios, containing 1 mL of an 8 mM solution of hemin in DMSO, 1 mL of complex 1 in deionized water, and 2 mL of 8 M acetate buffer (pH 5), were prepared (solutions of the inhibitor 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 to remove unreacted hematin, centrifuged again to separate the soluble fraction, and finally the pellet of pure β -hematin was dissolved in NaOH (0.1 M) for spectrophotometric quantification. A control experiment using CQDP was carried out under analogous conditions.

Heme aggregation inhibition at an *n*-octanol-acidic buffer interface

Inhibition of the heme aggregation at the interface of *n*-octanol-aqueous buffer mixtures was studied following a modification of the method described by Egan et al. [36]. To establish a base line 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 per milliliter. A sample of this hematin solution (100 μ L) was carefully introduced close to the interface between *n*-octanol (2 mL) and the acetate buffer (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 30 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 filtration through a cellulose filter disk, washed with DMSO to remove unreacted hematin, and finally dissolved in 25 mL of 0.1 M NaOH for spectrophotometric quantification. The aggregation inhibition activity measurements were conducted in a similar way by adding appropriate

amounts of an aqueous CQDP solution (23 mM) or complex **1** (11.5 mM) to yield drug-to-hemin concentration ratios in the range 1-6.

Determination of partition coefficients

Distribution of CQ or complex **1** between *n*-octanol and water was studied by use of the stir-flask method [37-39]. A mixture of 10 mL of each solvent, each saturated in the other, was stirred for 30 min at the desired temperature after adding the right amount of the sample (approximately 0.7 mmol) to be analyzed. Once equilibrium had been reached, the organic and aqueous phases were separated and centrifuged and the concentration of drug in each phase was measured spectrophotometrically in order to determine values of $K_{ow} = [\text{drug}](\text{in octanol})/[\text{drug}](\text{in water})$.

Interaction of complex **1** with CT DNA by spectrophotometric titration

The binding constants for complex **1**-CT DNA interactions were determined by absorption titration at room temperature through the stepwise addition of a CT DNA solution (10 μL , 2.89 mM) to a cell containing complex **1** (2 mL, 12.5 μM), both in 10 mM phosphate buffer containing 50 mM NaClO_4 (pH 6.8). Absorption spectra were recorded at 330 and 343 nm and the titration was terminated when the intensity at those wavelengths did not change significantly upon further addition of DNA. The simple model often used [40,41] to calculate a single binding constant according to the equation $[\text{DNA}]/(\epsilon_a - \epsilon_f) = [\text{DNA}]/(\epsilon_b - \epsilon_f) + 1/[K_b(\epsilon_b - \epsilon_f)]$ did not provide a good fit of our data. However, an excellent fit for two binding constants, K_{b1} and K_{b2} , was obtained by using the Scatchard equation $r/C_f = K(n - r)$ for ligand-macromolecule interactions with noncooperative binding sites [42-45], where r is the number of moles of ruthenium complex bound to 1 mol of CT DNA (C_b/C_{DNA}), n is the number of equivalent binding sites, and K is the affinity of the complex for those sites. Concentrations of free (C_f) and bound (C_b) complex **1** were calculated from $C_f = C(1 - \alpha)$ and $C_b = C - C_f$, respectively, where C is the total ruthenium concentration. The fraction of bound complex (α) was calculated from $\alpha = (A_f - A)/(A_f - A_b)$, where A_f and A_b are the absorbances of free and fully bound complex **1** at the selected wavelengths, and A is the absorbance at any given point during the titration. The plots of r/C_f versus r give the binding constants K_b as the slopes of the graphs.

Interaction of complex **1** with CT DNA by fluorimetric titration

Excitation and emission wavelengths for complex **1** were set to 343 and 380 nm with bandwidths of 4 and 8 nm, respectively. Using standard right-angle emission optics, we recorded fluorescence intensity measurements using the photon counting mode and corrected for any fluctuations of the 450-W xenon arc lamp source by deflecting a portion of the excitation signal onto a separate photodiode. The measured intensity signals are thus reported in the ratio mode (S/R) as counts per second (S) measured on the emission photomultiplier over the photodiode current (R). For all fluorescence studies, the absorption of the sample at the wavelength of excitation was less than 0.1 in order to obviate inner filter effects. The fluorimetric titration was carried out at room temperature. Fixed amounts (2 μL) of a 65 μM solution of CT DNA were added to a 2.5 μM solution of the metal complex, both in cacodylate buffer (10 mM cacodylate plus 100 mM KCl; pH 7.2), and emission spectra were monitored at 380 and 485 nm until saturation was reached. Binding data were cast into the form of a Scatchard plot of r/C_f versus r in a similar way as for absorption titration. In this case, α was calculated according to $\alpha = (F_f - F)/(F_f - F_b)$, where F_f and F_b are the fluorescence intensities of free and fully bound complex **1** at the selected wavelength, and F is the fluorescence intensity at any given point during the titration [46]. K_b is obtained from the slope of the plot.

Interaction of complex **1** with CT DNA by thermal denaturation experiments

Melting curves were recorded in media containing 50 mM NaClO₄ and 5 mM Tris-HCl buffer (pH 7.29). The absorbance at 260 nm was monitored for solutions of CT DNA (70 μM) before and after incubation with a solution of the drug under study (35 μM in Tris-HCl buffer) for 1 h at room temperature. The temperature was increased by 0.5 °C min⁻¹ between 65 and 82 °C and by 3 °C min⁻¹ between 25 and 65 °C and between 82 and 97 °C.

Results and discussion

Since its introduction during World War II, CQ had been the drug of choice for the treatment of malaria, owing to its high efficacy, low toxicity, and low price. However, the alarmingly rapid spread of drug-resistant malaria is of great concern and it appears that for the first time in 300 years there is a danger of not having effective quinoline-based drugs for the fight against malaria [10]. Relatively simple modifications of the molecular structure of CQ led to the development of promising drugs like amodiaquine, mefloquine, and halofantrine but, unfortunately, resistance to these drugs is now also common in many regions of the planet. On the other hand, introducing a metal-containing fragment into the structure of CQ produces a more dramatic modification in terms of the electronic structure and the physicochemical properties of the molecule and makes a number of alternative chemical and biochemical pathways available as part of a potential mechanism of antimalarial action; metal-DNA binding, for instance, is a common phenomenon that could lead to new or additional pathways of antimalarial action. Also, the basicity of the molecule as well as its hydrophilicity/lipophilicity characteristics are strongly modified by a metal-ligand array and this may result in improvements in the efficacy of the metal-based drug with respect to the parental organic compound, as demonstrated in the case of the iron-containing ferroquine [34]. Moreover, recognition of CQ by the parasite may be seriously impaired by the presence of the transition-metal-containing moiety, possibly leading to a reduction of resistance [21,22].

In our previous work with complex **1**, we found that the in vitro activity against the CQ-resistant Colombian FCB1 and FCB2 strains of *P. falciparum* was about 5 and 2.5 times higher than that of CQDP, respectively, at equivalent concentrations. Furthermore, at doses equivalent to the ED₅₀ of CQDP, complex **1** inhibited 94% of the induced parasitemia of mice infected with *P. berghei* with no accompanying signs of acute toxicity [28]. In order to ascertain the origin of these important effects, we have now studied some physicochemical characteristics of this metal complex and evaluated its heme aggregation inhibition activity and DNA binding ability in comparison with CQDP. The combined results shed light on the possible mechanism of action, in particular against CQ-resistant parasites.

Hydrolysis of complex **1**

The molecular structure of complex **1** was previously deduced by detailed NMR spectroscopic studies [28]. One interesting feature of this structure is that CQ is present in its unprotonated form and it coordinates to two separate “RuCl₂” units, effectively blocking both protonable nitrogen atoms. As expected, this leads to a marked decrease in the basicity of the molecule; the pH of a 1 mM aqueous solution of complex **1** is 6.3, whereas the value for a 1 mM solution of CQDP is 5.2. The electrical conductivity of a 1 mM aqueous solution of complex **1** rapidly (in less than 2 min) attains a value of 295 μS cm⁻¹, typical for three ions in solution, suggesting a fast exchange of one chloride by water from each ruthenium atom, as depicted in Scheme 1. Since Ru(II) complexes are almost always octahedrally hexacoordinated in solution, we propose the dicationic derivative [RuCl(OH₂)₃(CQ)]₂[Cl]₂ (**2**; Scheme 1) containing triaquated Ru(II) ions binding to CQ as the predominant species formed when complex **1** is dissolved in water. In contrast, the conductivity of a 1 mM solution of complex **1** in dimethylformamide is very low (32 μS cm⁻¹) and it remains constant over a 24-h period, indicating little or no

dissociation of the chloride ligands in nonaqueous media. The ^1H NMR spectrum of a D_2O solution of complex **1** remains unchanged for up to 24 h at room temperature, indicating that the solutions are stable under those conditions; addition of 0.1 M NaCl did not cause any spectral changes over a further 24-h period, suggesting that dissociation of the chlorides is not reversed by concentrations typical of extracellular or intracellular chloride. In order to avoid confusion, in the remainder of this paper we will refer to the complex under study as complex **1**, even if species **2** is likely to be the major one present in solution.

Partition coefficient measurements as a function of pH

In order to probe hydrophilicity/lipophilicity changes induced in CQ by coordination to ruthenium, the partition coefficients of CQDP and of complex **1** were measured in *n*-octanol-water mixtures at different mixture compositions, temperatures, and pH. The concentration of complex **1** in the octanol phase increases with increasing proportion of octanol and with increasing temperature (data not shown). More interestingly, the partition data for complex **1** as a function of pH, shown in Fig. 2 in comparison with CQDP, are much more informative. Plots of $\log K_{\text{ow}}$ versus pH allow a direct visualization of the accumulation of each drug in each phase, where $\log K_{\text{ow}} = 0$ represents equal partitioning between the two phases. Values of $\log K_{\text{ow}} < 0$ indicate accumulation in the aqueous phase, whereas $\log K_{\text{ow}} > 0$ corresponds to accumulation in the alcohol phase. CQ, as expected, displays a very strong hydrophilicity at pH values below 6 and a more lipophilic character at pH 6 and above, where it begins to deprotonate (Fig. 2a).

Complex **1**, on the other hand, displays a more strongly lipophilic character even at pH values close to 5 (Fig. 2b), indicating that in the pH region corresponding to the acidity of the food vacuole of the malaria parasite CQ will be concentrated strongly in the aqueous regions, whereas the effective concentration of complex **1** will be greater at or near water-lipid interfaces. This is important in connection with the proposal that lipids catalyze heme aggregation, probably by increasing heme solubility in acidic environments [47]; neutral lipids that catalyze the formation of hemozoin *in vitro* have been shown to form lipid bodies associated with plasmodium food vacuoles [48]. Egan et al. [36] recently demonstrated that under acidic physiologically realistic conditions β -hematin spontaneously forms by rapid self-assembly near octanol-water, pentanol-water, or lipid-water interfaces. Our partition data (Fig. 2) indicate that there will be higher concentrations of complex **1** near such interfaces under acidic conditions similar to those of the *Plasmodium* food vacuole and thus it should be a better heme aggregation inhibitor than CQ, which concentrates in the aqueous phase away from lipids. To further probe this important issue, we also evaluated the interaction of complex **1** with hematin and the heme aggregation inhibition activity of complex **1** in comparison with that of CQDP, in aqueous media and in acidic octanol-water interfaces, as described in the following section.

Interaction of complex **1** with hematin

The interaction of complex **1** with hematin was followed by spectrophotometric titration at the Soret band at 402 nm in aqueous DMSO at pH 7.4 and 4.97 (Fig. 3). Biot et al. [34] have previously described an extensive investigation of the interaction of Fe(III)PPIX with CQ; several possibilities were considered but a simple 1:1 association model fitted their data adequately, except at very low drug-to-hematin ratios. Following that procedure and data fitting for a 1:1 association model (with omission of the first few data points) [34], we measured values of $\log K = 5.37 \pm 0.01$ (at pH 7.4) and 5.01 ± 0.01 (at pH 4.97) for CQDP, in good agreement with the values reported by Biot et al. of 5.52 ± 0.03 at pH 7.5. The data for complex **1** were fitted in a strictly analogous manner to yield values of $\log K = 5.06 \pm 0.01$ at pH 7.4 (Fig. 3, inset; for further details see “Materials and methods”) and 4.81 ± 0.09 at pH 4.97 (data

not shown). This indicates that the metal-CQ complex interacts with hematin to an extent comparable to that of CQDP under these conditions.

Heme aggregation inhibition activity of complex 1 in acetate buffer and in an *n*-octanol-acetate buffer interface

A number of heme aggregation inhibition activity assays have been proposed and discussed in the literature. By using a variation of the β -hematin inhibition activity method reported by Parapini et al. [35], we measured the ability of CQDP and of complex **1** to inhibit the formation of β -hematin after 24 h under strictly comparable conditions, starting from commercial hemin in acetate buffer (pH 5). As can be seen from the data in Fig. 4a, the difference between CQDP and complex **1** under these conditions is not very marked, with CQDP being somewhat more active than complex **1** (50% inhibition was observed at a drug-to-hemin concentration ratio of about 0.8 for CQDP and about 1.0 for complex **1**). However, as shown in Fig. 4b, when the inhibition assay was conducted in a 1:1 *n*-octanol-water mixture, a simple model of a water-lipid interface [49], following an adaptation of the procedure described by Egan et al. [36] in which both the hematin and the drug are carefully introduced close to the interface, the overall process is much faster (30 min), and more importantly, the activity trend is reversed and complex **1** was significantly more efficient than CQDP (50% inhibition at drug-to-hemin concentration ratios of approximately 1.2 and 2.0, respectively). These results, together with the observation of Egan et al. that β -hematin rapidly forms near octanol-water or lipid-water interfaces [36], led to the idea that a drug that concentrates near such interfaces at a pH value typical of the acidic food vacuole of *Plasmodium* parasites should be in a better position than the highly hydrophilic CQ to inhibit the process of heme aggregation, and this provides an explanation for the higher antimalarial activity of complex **1** compared with CQDP [28].

A possible explanation for the activity of complex 1 against CQ-resistant *P. falciparum*

Despite the fact that complex **1** appears to share its main target with CQ (heme aggregation), the metal derivative has shown enhanced potency against resistant strains of *P. falciparum* [28]. CQ resistance has been associated with polymorphisms within the *pfcr* gene but the actual mechanism by which drug accumulation is reduced is still the subject of much debate [16-19]. *pfcr* encodes a 48.6-kDa protein PfCRT, which is thought to function as a transmembrane transporter localized at the membrane of the digestive vacuole. Mutations in this protein are directly linked to CQ resistance, particularly K76T, which involves the change of lysine, a charged amino acid, to uncharged threonine. CQ binds to PfCRT and it has been suggested that because of the specific mutation involved, the ability of compounds to be extruded through PfCRT decreases with increasing lipophilicity [50,51]. Indeed it has been observed that a number of drugs that are active against CQ-resistant strains, including metal-based CQ derivatives like the iron-containing ferroquine [31,32,34] and the gold-CQ complex [Au(CQ)(PPh₃)₃]₃PF₆ reported by us [28], are considerably more lipophilic than CQ and this could be an important factor in the reduction of resistance. Also, a major structural modification of CQ as in complex **1** is likely to result in a lowered ability to interact with the transporter, which seems to be highly structure specific.

Is DNA binding important for the antimalarial action of complex 1?

DNA binding was considered the major mechanism of antimalarial action of CQ for many years in the early literature [12]. Although there have been some attempts to revive this hypothesis in recent times [52,53] and despite the fact that acridine-based and other related antimalarials are thought to exert their action predominantly or partially via DNA interactions [13-15], most of the experimental evidence available points toward heme aggregation as the main target for CQ to damage parasite cells. Nevertheless, the modification of CQ by attachment to a metal-containing fragment reported by us [21,22,28-30] could reopen the

possibility of DNA as a major or secondary target of therapeutic action, since metal complexes are known to interact with DNA in a variety of ways [20]. In order to probe this possibility, we investigated the interaction of complex **1** with DNA by use of different techniques and compared its behavior with that of CQDP under analogous conditions.

Figure 5 shows the results of a spectrophotometric titration of complex **1** with CT DNA in 10 mM phosphate buffer at pH 6.8. The bands at 330 and 343 nm, which correspond to CQ bound to ruthenium, both decrease in intensity with incremental addition of DNA, clearly indicating an interaction between complex **1** and DNA; from the Scatchard plot shown in Fig. 5 (inset, for details see “Materials and methods”) two binding constants could be calculated at 343 nm, consistent with two principal binding interactions [42-45]. Independent and consistent values for the two constants were determined from a similar plot at 330 nm (data not shown). Table 1 contains the averaged values for the two wavelengths.

This interaction was also studied by fluorimetric titration in 10 mM cacodylate buffer at pH 7.2. The emission band at 385 nm, originating from complex **1** (see Fig. 6), decreases in intensity with increasing concentration of added DNA. At the same time, a new band grows with incremental addition of DNA at about 480 nm, presumably owing to the formation of an adduct between complex **1** and DNA [54,55]. The corresponding Scatchard plot for data at 385 nm (Fig. 6, inset) provides values of K_{b1} and K_{b2} consistent with the ones calculated from absorption data. Independent values for the two constants could be obtained from a similar plot of data collected at 480 nm (not shown); average values of K_{b1} and K_{b2} for the two wavelengths are collected in Table 1. It can be noted that the binding constants obtained from fluorescence spectra are, within experimental error, in general agreement with the absorption measurements.

Under analogous conditions, CQDP interacts with DNA in a similar way, providing two binding constants with values comparable to those obtained for complex **1** (see Table 1) and we therefore conclude that the interaction of complex **1** with CT DNA is essentially analogous to that of free CQ. It has been known for some time that CQ binds to DNA by intercalation involving at least two kinds of interaction, one of electrostatic nature between the positively charged diethylammonium side chain and negatively charged phosphate residues in the nucleic acid polymer, and a second one, dependent on the secondary structure of DNA [12]. Nevertheless, a strong argument against DNA binding being the mechanism of CQ action is that the concentrations required to produce toxicity associated with DNA interactions are at least 1,000 times higher than the ones associated with antimalarial effects [8-10].

In order to rule out other possible interactions between complex **1** and DNA involving covalent bonding between the metal and the nucleobases, the nature of the interaction with DNA was further probed by thermal denaturation experiments. As depicted in Fig. 7, the melting point of CT DNA was increased by 3.4 °C after incubation with complex **1** for 1 h in 5 mM Tris-HCl buffer at pH 7.29, whereas incubation with CQDP under analogous conditions produced an increase of 1.8 °C (data not shown); both values are consistent with an intercalation mode of binding [56]. For comparison, Fig. 7 also shows the melting curve for DNA after incubation with the widely used anticancer drug cisplatin [*cis*-diamminedichloroplatinum(II)], which is known to bind strongly to DNA through covalent interactions between the metal and guanine bases [57]; in this case, a decrease in the melting temperature of 7.2 °C was observed.

These combined results indicate that the attachment of the metal-containing fragment to CQ in complex **1** does not alter the DNA binding properties of CQ to any significant extent and therefore we conclude that the interaction that takes place between complex **1** and DNA is not an important component of the mechanism of antimalarial action.

Conclusions

The possible mechanisms of antimalarial action of $[\text{RuCl}_2(\text{CQ})]_2$ (**1**), which we previously reported to be active in vitro against CQ-resistant strains of *P. falciparum* and in vivo against *P. berghei* [28], have been investigated by a variety of techniques. In aqueous solution, the complex is rapidly transformed into the dicationic derivative $[\text{RuCl}(\text{OH}_2)_3(\text{CQ})]_2[\text{Cl}]_2$ (**2**), which is probably the active species. In spite of the presence of a transition metal in the molecular structure, complex **1** binds to DNA most likely through intercalation of the coordinated CQ moiety, very similarly to the mode of binding of free CQ to DNA, and therefore this does not appear to be an important part of the mechanism of antimalarial action. On the other hand, the heme aggregation inhibition activity of complex **1** is significantly higher than that of CQDP at the interface of *n*-octanol-aqueous acetate buffer mixtures at pH ~ 5, but is slightly lower in homogeneous acidic aqueous buffer. This suggests that heme aggregation is the principal target of the ruthenium-CQ complex **1** and it is important to also note that complex **1** is less basic and more lipophilic than CQ and its concentration must therefore be higher near the interface of *n*-octanol-water mixtures under acidic conditions that model the food vacuole of the parasite. This means that the metal complex probably accumulates near water-lipid interface regions within the digestive vacuole of the parasite, rather than deeply inside the aqueous regions where CQ concentrates. This observation, together with the idea that lipids catalyze heme aggregation within plasmodium food vacuoles [47,48] and with the findings of Egan et al. [36] that β -hematin spontaneously forms by rapid self-assembly near octanol-water or lipid-water interfaces can explain the enhanced antimalarial activity of complex **1** both in vitro and in vivo, when compared with CQ. Furthermore, the fact that the complex is active against CQ-resistant strains of *P. falciparum* may also be related to its greater lipophilicity, in line with previous reports indicating a lowered ability of the mutated transmembrane transporter PfCRT to promote the efflux of highly lipophilic drugs [40,51].

Acknowledgments

Financial support from the NIH through grants no. 1S06 GM 076168-04 (to R.A.S.-D) and 1S06 GM 076168-01 (to L.D.) is gratefully acknowledged.

References

1. <http://www.who.int/mediacentre/factsheets/fs094/en/index.html> (WHO Fact Sheets)
2. Sachs J, Malaney P. Nature 2002;415:680–685. [PubMed: 11832956]
3. Winstanley PA. Parasitol Today 2000;16:146–153. [PubMed: 10725901]
4. Rosenthal, PhJ, editor. Antimalarial chemotherapy: mechanisms of action, resistance and new directions in drug discovery. Humana Press; New Jersey: 2001.
5. Weisner J, Ortmann R, Jomaa H, Schlitzer M. Angew Chem Int Ed 2003;42:5274–5293.
6. Kumar S, Guha M, Choubey V, Maity P, Bandyopadhyay U. Life Sci 2007;80:813–828. [PubMed: 17157328]
7. Wellems TE. Science 2002;298:124–127. [PubMed: 12364789]
8. Egan TJ, Marques HM. Coord Chem Rev 1999;190-192:493–517.
9. Ziegler J, Linck R, Wrigth DW. Curr Med Chem 2001;8:171–189. [PubMed: 11172673]
10. Tilley, L.; Loria, P.; Foley, M. Antimalarial chemotherapy: mechanisms of action, resistance and new directions in drug discovery. Rosenthal, PhJ, editor. Humana Press; New Jersey: 2001. p. 87-121.
11. Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. Nature 2000;378:138–139.
12. Cohen SN, Yielding KL. Proc Nat Acad Sci 1965;54:521–527. [PubMed: 5324393]
13. Guetzoian L, Ramiandrasoa F, Dorizon H, Desprez C, Bridoux A, Rogier C, Pradines B, Perrée-Fauvet M. Bioorg Med Chem 2007;15:3278–3289. [PubMed: 17339112]
14. Dheyongera JP, Geldenhuys WJ, Dekker TG, Matsabisa MG, Van der Schyf CJ. Bioorg Med Chem 2005;13:1653–1659. [PubMed: 15698783]

15. Van Miert S, Jonckers T, Cimanga K, Maes L, Maes B, Lemièrre G, Dommissie R, Vlietinck A, Pieters L. *Exp Parasitol* 2004;108:163–168. [PubMed: 15582513]
16. Dorsey, G.; Fidock, DA.; Wellems, TE.; Rosenthal, PhJ. Antimalarial chemotherapy: mechanisms of action, resistance and new directions in drug discovery. Rosenthal, PhJ, editor. Humana Press; New Jersey: 2001. 2001. p. 153-172.
17. Sanchez C, McLean JE, Rohrbach P, Fidock DA, Stein WD, Lanzer M. *Biochem* 2005;44:9862–9870. [PubMed: 16026158]
18. Sanchez C, McLean JE, Stein WD, Lanzer M. *Biochem* 2004;43:16365–16373. [PubMed: 15610031]
19. van Schalkwyk DA, Egan TJ. *Drug Resist Updat* 2006;9:211–226. [PubMed: 17064951]
20. Siegel, H.; Siegel, A., editors. Metal ions in biological systems. 41. Marcel Dekker; New York: 2004. Metal ions and their complexes in medication.
21. Sánchez-Delgado RA, Anzellotti A. *Mini Rev Med Chem* 2004;4:23–30. [PubMed: 14754440]
22. Sánchez-Delgado, RA.; Anzellotti, A.; Surez, L. Metal ions and their complexes in medication. In: Siegel, H.; Siegel, A., editors. Metal ions in biological systems. 41. Marcel Dekker; New York: 2004. p. 379-419.
23. Sánchez-Delgado RA, Lazzardi K, Rincón L, Urbina JA, Hubert AJ, Noels AN. *J Med Chem* 1993;36:2041–2043. [PubMed: 8336342]
24. Sánchez-Delgado RA, Navarro M, Lazzardi K, Atencio R, Capparelli M, Vargas F, Urbina JA, Bouillez A, Noels AF, Masi D. *Inorg Chim Acta* 1998;275-276:528–540.
25. Navarro M, Lehmann T, Cisneros EJ, Fuentes A, Sánchez-Delgado RA, Silva P, Urbina JA. *Polyhedron* 2000;19:2319–2325.
26. Navarro M, Cisneros-Fajardo E, Lehmann T, Sánchez-Delgado RA, Atencio R, Silva P, Lira R, Urbina JA. *Inorg Chem* 2001;40:6879–6884. [PubMed: 11754267]
27. Strasberg-Rieber M, Anzellotti A, Sánchez-Delgado RA, Rieber M. *Int J Cancer* 2004;112:376–384. [PubMed: 15382061]
28. Sánchez-Delgado RA, Navarro M, Pérez H, Urbina JA. *J Med Chem* 1996;39:1095–1099. [PubMed: 8676344]
29. Navarro M, Pérez H, Sánchez-Delgado RA. *J Med Chem* 1997;40:1937–1939. [PubMed: 9191972]
30. Navarro M, Vásquez F, Sánchez-Delgado RA, Pérez H, Sinou V, Schrevel J. *J Med Chem* 2004;47:5204–5209. [PubMed: 15456263]
31. Biot C. *Curr Med Chem Anti-Infect Agents* 2004;3:135–147.
32. Biot C, Daher W, Ndiaye CM, Melnyk P, Pradines B, Chavain N, Pellet A, Fraisse L, Pelinski L, Jarry C, Brocard J, Khalife J, Forfar-Bares I, Dive D. *J Med Chem* 2006;49:4707–4714. [PubMed: 16854077]
33. Egan TJ, Mavuso WW, Ross D, Marques HM. *J Inorg Biochem* 1997;68:137–145. [PubMed: 9336973]
34. Biot C, Taramelli D, Forfar-Bares I, Maciejewski LA, Boyce M, Nowogrocki G, Brocard JS, Basilico N, Olliaro P, Egan TJ. *Mol Pharm* 2005;2:185–193. [PubMed: 15934779]
35. Parapini S, Basilico N, Pasini E, Egan TJ, Olliaro P, Taramelli D, Monti D. *Exp Parasitol* 2000;96:249–256. [PubMed: 11162378]
36. Egan TJ, Chen JY-J, de Villiers KA, Mabotha TE, Naidoo KJ, Ncokazi KK, Langford SJ, McNaughton D, Pandiancherri S, Word BR. *FEBS Lett* 2006;580:5105–5110. [PubMed: 16956610]
37. OECD Guidelines for testing of chemicals. OECD; Paris: 1995.
38. Danielsson LG, Zhang YH. *Trends Anal Chem* 1996;15:188–196.
39. Rappel C, Galanski M, Yasemi A, Habala L, Keppler B. *Electrophoresis* 2005;26:878–884. [PubMed: 15714548]
40. Wolf A, Shimer GH Jr, Meehan T. *Biochemistry* 1987;26:6392–6396. [PubMed: 3427013]
41. Navarro M, Hernández C, Colmenares I, Hernández P, Fernández M, Sierralta A, Marchán E. *J Inorg Biochem* 2007;101:111–116. [PubMed: 17055060]
42. McGhee JD, Von Hippel PH. *J Mol Biol* 1974;86:469–489. [PubMed: 4416620]
43. Wei C, Jia G, Yuan J, Feng Z, Li C. *Biochemistry* 2006;45:6681–6691. [PubMed: 16716079]

44. Boyer, R. Benjamin Cummings, Reading. 2006. Biochemistry laboratory: modern theory and techniques; p. 253-260.
45. Cusumano M, Di Pietro ML, Giannetto A. Inorg Chem 1999;38:1754–1758. [PubMed: 11670943]
46. Satyanarayana S, Dabrowiak JC, Chaires J. Biochem 1992;31:9319–9324. [PubMed: 1390718]
47. Corrêa Soares JBR, Maya-Monteiro CM, Bittencourt-Cunha PRB, Atella GC, Lara FA, d'Avila JCP, Menezes D, Vannier-Santos MA, Oliveira PL, Egan TJ, Oliveira MF. FEBS Lett 2007;581:1742–1750. [PubMed: 17418143]and references therein
48. Jackson KE, Klonis N, Ferguson DJ, Adisa A, Dogovski C, Tilley L. Mol Microbiol 2004;54:109–122. [PubMed: 15458409]
49. Benjamin I. Chem Phys Lett 2004;393:453–456.
50. Warhurst DC. Malaria J 2003;2:31–43.
51. Warhurst DC, Craig JC, Adagu IS, Meyer DJ, Lee SY. Malaria J 2003;2:26–30.
52. Meshnick SR. Parasitol Today 1990;6:77–79. [PubMed: 15463303]
53. Li G-D. Med Hypotheses 2006;67:323–326. [PubMed: 16549276]
54. Wang Y, Yang ZY. Trans Med Chem 2005;30:902–906.
55. Satyanarayana S, Dabrowiak JC, Chaires J. Biochem 1992;31:9319–9324. [PubMed: 1390718]
56. Novakova O, Chen H, Vrana O, Rodger A, Sadler PJ, Bravec V. Biochem 2003;42:11544–11554. [PubMed: 14516206]
57. Farrell, N. Transition metal complexes as drugs and chemotherapeutic agents. Dordrecht; Kluwer: 1989.

Fig. 1.
Structure of chloroquine (CQ)

Fig. 2. Partition coefficients for **a** chloroquine diphosphate (CQDP) and **b** [RuCl₂(CQ)]₂ (**1**) in a 1:1 *n*-octanol-water mixture

Fig. 3.
Interaction of complex **1** with hematin at pH 7.4

Fig. 4. Heme aggregation inhibition at different concentrations of CQDP (*filled circles*) and complex **1** (*open circles*) in **a** acetate buffer at pH 5 after 24-h incubation at 37°C and **b** *n*-octanol-acetate buffer at pH 4.9 (2:5 by volume) after 30-min incubation at 37 °C

Fig. 5. Spectrophotometric titration of complex **1** with calf thymus (CT) DNA in 10 mM phosphate buffer, pH 6.8. *Inset:* Scatchard plot of data at 343 nm used to calculate values of K_{b1} and K_{b2} from linear regression of the data (*filled circles* and *open circles*, respectively)

Fig. 6. Fluorimetric titration of complex **1** with CT DNA in 10 mM cacodylate buffer at pH 7.2. *Inset:* Scatchard plot of data at 480 nm used to calculate values of K_{b1} and K_{b2} from linear regression of the data (*filled circles* and *open circles*, respectively). See Table 1

Fig. 7. Melting curves for free CT DNA (*dashed line*) and CT DNA after incubation with complex **1** (*dashed-dotted line*) or cisplatin (*solid line*) for 1 h in 5 mM tris(hydroxymethyl) aminomethane hydrochloride buffer at pH 7.29. *Inset:* First derivatives of melting curves showing maxima at 79.7, 83.1, and 72.5 °C, respectively. The corresponding value for CT DNA after incubation with CQDP was 81.5 °C

Scheme 1.
Hydrolysis of complex **1**

Structure 1.

Table 1
Binding constants for the interaction between $[\text{RuCl}_2(\text{CQ})]_2$ (**1**) and calf thymus DNA

Compound	K_{b1} ($\times 10^7 \text{ M}^{-1}$) ^a	K_{b2} ($\times 10^5 \text{ M}^{-1}$) ^a	K_{b1} ($\times 10^7 \text{ M}^{-1}$) ^b	K_{b2} ($\times 10^5 \text{ M}^{-1}$) ^b
$[\text{RuCl}_2\text{CQ}]_2$	2.04 ± 0.69	1.80 ± 0.07	5.29 ± 1.43	6.02 ± 2.97
CQDP	1.07 ± 0.05	1.23 ± 0.68	2.63	4.38

CQ chloroquine, CQDP chloroquine diphosphate

^a Averages of values calculated at 330 and 343 nm from absorption spectra, measured in 10 mM phosphate buffer containing 50 mM NaClO₄, pH 6.8

^b Averages of values calculated at 385 and 480 nm from fluorescence spectra, measured in 10 mM cacodylate buffer containing 100 mM KCl, pH 7.2