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Correlation Assay Between Bioactivity and Thermodynamic Stability of Hemin Complexes of Some Quinoline Antimalarial Based-drugs

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Abstract

Malaria is a serious public health problem in developing countries and affects about 500 million people per year. However, resistance of some plasmodial strains to the chemotherapy effect of quinoline based-antimalarial drugs has caused the resurgence of malaria in endemic areas. The aim of this study was to evaluate the antimalarial activity of complexes of hemin with chloroquine, amodiaquine, quinine, quinidine and mefloquine. The complexes of hemin with chloroquine, amodiaquine, quinine, quinidine and mefloquine were prepared *in vitro*, using water-propylène glycol 30% mixture as solvent. The *in vitro* antimalarial assays were carried out according to WHO protocol and expressed as IC_{50} i.e concentration of drug which kills 50% of the parasite. The *in vivo* antiplasmodial activity was evaluated by thick smear while the average weights of mice before and after the exposure to the hemin-amodiaquin complex were used to evaluate the complex toxicity. The IC_{50} shows a relatively high activity for all complexes tested, in comparison with the corresponding quinoline antimalarial based drug alone ($p<005$). The *in vivo* antimalarial assay with hemin-amodiaquine complex (H-AQ) shows a total disappearance of parasitaemia at day 7 after infection of Balb/c mice with *P. berghei*. The preliminary toxicity test on mice has revealed that H-AQ is not toxic. Besides, any correlation was found between the antimalarial activity and the thermodynamic stability considering all complexes. This could be explained by the great discrepancy observed in susceptibility of used parasite strains. It is therefore desirable that the antiplasmodial test be carried on standardized strains using the isotopic test. It would be interesting to include other quinoline derivatives and corresponding complexes in order to confirm the thermodynamic-biological activity correlation of complexes by determining an area of temperature, pH, ionic strength, etc. to which the complexation constant would respect the order of parasitological activity.

Keywords

Malaria, Quinoline Antimalarial Based Drugs, Ferriprotoporphyrin IX, Complex, Thermodynamic Stability

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1. Introduction

Malaria is a serious public health problem in developing countries and affects about 500 million people per year [1]. In order to combat this scourge, various antimalarial products are used and some products such as quinine and chloroquine, quinoline-based compounds, have shown a great antimalarial activity. However, resistance of some plasmodial strains to the chemotherapy effect of antimalarials has caused the resurgence of malaria in endemic areas. This malaria

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resistance is a real calamity for Africa because it mainly affects quinoline antimalarial based-drugs particularly chloroquine, a molecule easily accessible geographically and financially which could be combining with hemin to potentiate its bioactivity.

The fear of losing these antimalarials justifies the current renewed interest in understanding their mode of action. A better understanding of this mode would allow us to understand the mechanism of antiplasmodial resistance associated with their use on the one hand, and to produce new molecules more effective or improve the activity of antimalarial molecules available on the other hand. Several experimental evidence highlighted the crucial role played by hemin in the therapeutic activity of antimalarials, both quinoline and non-quinoline [2-9]. In this mechanism, the hemin or ferriprotoporphyrin IX [Fe (IX) PP] acts as receptor of antimalarials with quinoline core (APQ) with which it forms complexes [10]. The latter would be responsible for lysis of the vacuolar membrane and thus cytotoxicity of the AQPs to the parasites. Hemin is thought to result from the degradation of hemoglobin in the host cell by the parasite. This metabolic waste is toxic to the parasite but it is detoxified by its conversion into an insoluble crystalline pigment, hemozoin, of which subunits are united by the coordination link between ferric iron (Fe^{+++}) and radical carboxylate [11]. In the human host, the presence of hemozoin would induce the formation of nonenal trans-4-hydroxy-2 from the peroxidation of membrane lipids. This cytotoxic compound, considered as a second messenger in the action of free radicals, is believed to be involved in neuron apoptosis and macrophages as well as in the suppression of cytokine biosynthesis pathways [12-16]. Because hemozoin formation is a unique and specific process of the parasite, it is now a pharmacological target of choice intensively exploited during the screening of high-potential antimalarial compounds [17, 18]. Indeed, the complexation of hemin with antimalarials in the digestive vacuole of the parasite would therefore prevent its bio-hemozoin crystallization.

In this work, we studied the *in vitro* antimalarial activity of hemin complexes with chloroquine, amodiaquine, quinine, quinidine and mefloquine. The relevance of this study is obvious, first of all in the case of a proven effectiveness of all complexes compared to APQs alone, and then further evidence would be provided to the theory of hemin [6]. Second, the study would determine whether there is a possible correlation between thermodynamic stability and pharmacological activity of hemin and antimalarial complexes. Such a correlation could be used to predict the antimalarial activity of new molecules from their complexation constants. Indeed, complexation constants can be determined simply by many less expensive physical methods such as UV-visible spectroscopy.

This would significantly reduce the costs associated with *in vitro* parasitological tests and encourage the production of these molecules.

2. Materials and Methods

2.1. *Preparation* of Stock Solutions

The stock solution of hemin (sigma p. a product) 400 µM is prepared by dissolving the hemin in a volume of alcalinized bi-distilled water (pH-9) to which is added the propylene glycol PGOH (sigma p. a product) 30% (v/v) propylene glycol mixture. The APQ stock solutions 2mM are prepared by dissolving chloroquine (CQ), mefloquine (MQ), quinine (CQ) and quinidine (QD) (SIGMA p. a products) in acidified bi-distilled water (pH 4) while amodiaquine (AQ) is directly dissolved in bi-distilled water around pH 7 before the addition of PGOH.

2.2. *Complex* Solution

2.2.1. Theoretical Treatment

Considering a simple pattern of complexation where a hemine molecule (H) reacts with an antimalarial molecule (A), the complexation balance can be written according tothe following equation:

$$H + A \xleftrightarrow{K} HA \quad (1)$$

In diluted solution, the K-balance constant depends on the ratio of reagents' concentrations to the complex formed according to the following relationship:

$$K = \frac{C_{HA}}{C_{HC_A}} \; where \; K = \frac{1}{K_d} \quad (2)$$

Where K_d is to the dissociation constant. Assuming that CHA-Y is the fraction of hemin saturated in A i.e. the complex and $C_H = 1 - Y$ which is the fraction of the free hemin. It can be shown that

$$Y = \frac{1}{1+\frac{K_d}{C_A}} \quad (3)$$

To avoid the problem of oversaturation of hemin in antimalarials, the complex (Y) rate was set at 50%.

2.2.2. Preparation of the Complex

The Fe (IX) PP-APQ complex is achieved by mixing well-known volumes of the stock solutions of hemin and antimalarials in order to obtain a concentration of about 20 µM for hemin and 62.5; 30; 35.7; 2.3 and 476 µM for AQ, CQ, Q, Qd and MQ respectively. The volume obtained is

adjusted with the water-PGOH 30% mixture. Indeed, in conditions where the concentration of APQ is equivalent to the value of the constant of dissociation of the complex to balance (in equilibrium), all the antimalarial is then complexed. All solutions are buffered with Tris (hydroxymethylyl) aminomethane (product ALDRICH p. a). Concentrated solutions from NaOH and HCl (MERCK p. a product) were used, as appropriate, to adjust the pH. The IKAMAG REO magnetic agitator has been used to homogenize solutions.

All pH measurements were made using a METROHM 604 pH-meter with a combined glass electrode, previously calibrated using standard solutions and kept in a KCl 3M water solution. After each measurement, the electrode is carefully rinsed with bi-distilled water.

2.3. *Biological* Tests

2.3.1. In vitro Test

i. *Plasmodial* strains and inclusion criteria

Plasmodium falciparum isolates were obtained from impaled blood from patients who came to the clinic and examined at the Kindele/Mont Ngafula Health Centre in Kinshasa. The choice was made for the patient who had a high parasitemia (above 90 trophozoites per microscopic field) and who had not received antimalarial treatment in the two weeks before the parasitological diagnosis. Blood samples were taken by verbal consent of the parents or guardians accompanying the selected children (4 to 8 years old).

ii. *The in vitro* antiplasmodial test

The sterile glass microtest plates were impregnated with different drug solutions at different concentrations due to 5 µL of each solution per bucket. These extracts were then evaporated to dryness in an oven (Jouan brand) at 37°C for two days. The malarial culture medium was made by diluting 1 ml of malarial blood in 9 ml of culture medium RPMI 1640 supplemented with 25 mM HEPES buffer N-(2-hydroxythyl) pipazine-N'-(2-ethane sulfonate) and 30 mM of $NaHCO_3$ (10:1:1 (v/v/v). The antipaludogram is performed by spotting 45 µl of the malarious culture medium in each bucket of the sterile microtest plates previously impregnated with the drug extracts as previously described. The plates are covered with parafilm paper and placed in a candle bell emptied of oxygen by combustion. The incubation was done at 37°C for 42 hours. After incubation, the contents of each bucket are processed using the technique of confection and staining of the thick drop and examined by an LC 100 optical microscope. The number of asexual parasites (trophozoites and erythrocytic schizonts) was determined using a hand counter. The percentages of maturation of trophozoites as well as those of inhibition of the maturation of trophozoites were subsequently calculated. Inhibitory concentrations 50 (CI_{50}) are calculated based on linear regression analysis of dose-effect pairs using Origin 6.1 software.

2.3.2. The *in Vivo Antiplasmodial* and Toxicological Tests

i. *The in vivo* antiplasmodial test

Plasmodium berghei (ANKA strain) was reconstructed by the PBS buffer (8 g NaCl; 0.2 g KCl; 0.2 g $CaCl_2$; 0.3 g KH_2PO_4; 0.8 g Na_2HPO_4; 2.5 g glucose; pH 7.2). Then, 300 µL of this parasitic suspension were inoculated intra-peritonally (IP) to 5 mice Balb/c while 200 µL of blood impaludated with P. (V.) berghei obtained from the best responder (mice with a high parasitaemia) were subsequently inoculated IP along with 15 other mice (day zero, D_0). After 72 hours following the inoculation, the infection was confirmed by thick smear (TM) then these rodents were immediately divided into three batches of 5 mice (test group T_1, standard group T_2 and control group T_0). The hemine-amodiaquine complex H-AQ (70 nmoL/L: 0.5 mL/day), chloroquine (250 mg/Kg) and bi-distilled water (0.5 mL/day) were administered orally for three consecutive days (D_1, D_2 and D_3) to different experimental as follows T_1, T_2 and T_0. The parasitemia was assessed on days D_1, D_4 and D_7 in treated and non-treated animals with TM.

ii. *Toxicological* test

Three experimental batches of 5 male Balb/c mice aged more or less 3 months (test group T_1, control group 1 (T_{01}) and control group 2 (T_{02}) were formed. The H-AQ complex (1.6 µM/L: 0.5 mL/day), the bi-distilled water (0.5 mL/day) and the water-PGOH 30% mixture (0.5 mL/day) were administered three times a week at T_1, T_{01} and T_{02} by gavage using 1cc insulin syringes. Mice were observed for two weeks during which they have free access to food. The weights of the mice were noted at the beginning/baseline (D_0) and at the end of the experiment (D_{14}) using an OHAUSS brand electric scale.

3. Results and Discussion

The evaluation of the antiplasmodial activity of chloroquine (CQ), amodiaquine (AQ), quinine (Q), quinidine (QD), mefloquine (MQ), hemin (H) and hemine-chloroquine complexes (H-CQ), hemine-amodiaquine (H-Aq), hemine-quinine (H-Q), hemine-quinidine (H-QD), hemine-mefloquine (H-MQ), are included in Tables 1 and 2 below.

Table 1. IC_{50} values of the quinoline antimalarial based drugs and corresponding complexes.

class	Drug	IC_{50} (nmol/L)
I.	CQ	135±7
	H-CQ	102±13
	AQ	118±13

class	Drug	IC_{50} (nmol/L)
II.	H-AQ	74±9
	Q	47±15
	H-Q	38±14
	QD	103±24
	H-QD	65±2
	Sqm	67±5
	H-MQ	54±5

(Legend: I: Class of amino-4-quinoines and corresponding complexes; II: Class of carbinol-4-quinoleins and corresponding complexes).

This table shows that all plasmodial strains tested are resistant to chloroquine, amodiaquine and mefloquine. These phenotypes can be highlighted from the concentrations values for which 50% of parasites are killed (IC_{50}). The CI_{50} of the aforementioned drugs are higher than 120, 70 and 50 nmol/L (critical value) respectively. On the other hand, these same plasmodial strains are sensitive to quinine (IC_{50}<500 nmol/L) [19]. The results of the same table also generally show that all plasmodial strains tested are sensitive to the hemin complex with APQs compared to the corresponding antimalarial molecules alone. In addition, within the amino-4-quinoinin class, the hemine-amodiaquine complex appears to be more effective than the hemin-chloroquine complex while in the carbinol-4-quinolein class it is the hemin-quinine complex that appears to be more active followed respectively by the hemin-mefloquine and hemine-quinidine complexes.

The superiority of the antiplasmodial activity of the complexes over antimalarials alone can be confirmed or reversed by statistical analysis. The statistical t-test test application to the data in Table 1 shows that the superiority of complex activity is not due to a chance or random dispersion.

Table 2. The inhibition Percentage of the trophozoites maturation by hemin compared to the control (water-propylene glycol mixture 30%, 0.1M Tris buffer, pH 7.40).

Sample	Concentration of Hemin (nmol/L)							
	6400	3200	1600	800	400	200	100	50
A	10	9	6	4	3	2	1	1
B	12	10	9	6	3	1	1	1
C	13	9	7	5	4	4	3	1
Average (%)	12	9	7	5	4	2	2	1

This table shows that for the same concentrations used to assess the antiplasmodial activity of the corresponding APQs and complexes, the rate of inhibition of trophozoite maturation by hemin does not exceed 12%.

Table 3 shows the evolution of malaria parasitemia in Balb/c mice infected with *P. berghe*i and treated with chloroquine 5 mg/kg or the hemine-amodiaquine complex 70 nmol/l.

Table 3. Evaluation of parasitemia in treated and untreated mice by thick smear.

Day	Thick drop		
	Control group	Test group	Standard group
D_0	-	-	-
D_1	+	+	+
D_4	+++	+	++
D_7	ND	-	++

Legend: -: absence of trophozoites in the blood (figure 2); +: 1-10 trophozoites by 100 microscopic fields; ++: 11-100 trophozoites by 100 microscopic fields; +++: 1-10 trophozoites by microscopic field (figure 1); ND: non-determined.

Table 3 shows that the treatment of mice with the hemin-amodiaquine complex 70 nmol/L eliminates parasitemia on D_7 after infection, while those treated with chloroquine have a charge about 11-100 trophozoites per 100 microscopic fields. This result indicates that the hemin-amodiaquine complex also possesses *in vivo* activity. An important activity of complexes both *in vitro* and *in vivo* can only be explained to the extent that the APQs act through the complexes they form with the hemin [6]. Indeed, if one accepts that APQs act through a mechanism that does not involve the formation of a complex, one should therefore expect that their interaction with hemin will make them less available and therefore less active. Furthermore, complexes should not show any antimalarial activity or, if it existed, it should be reduced from that of APQs alone. In addition, it should be noted that the activity of the complex may not be due to an excess of hemin because at the concentrations used, the activity of the hemin is insignificant (inhibition rate ≤12%).

This result seems contradictory for a molecule that is presumed to be toxic to plasmodial proteases [9, 20]. Indeed, it is known that hemin is highly toxic to parasites with high concentrations, in the range 200-400 mmol/L in the digestive vacuole (DV) more than 5.10^4 times the highest concentration used in this work. Thus, at low concentrations, the hemin would not be toxic to parasites. To reduce the vacuolary concentration of hemin and make it less toxic, *P. falciparum* would use three different metabolic pathways: (1) at the level of the digestive vacuole, part of the hemin would be degraded by hydrogen peroxide produced during the oxidation of the heme (a product of degradation of hemoglobin of the host cell) into hemin while (2) the other part would be converted to hemozoin or malaric pigment. At last, (3) the hemin would pass through the vacuolar membrane to the cytosol where it is destroyed following a mechanism involving glutathione activity. Moreover, the superiority of the activity of the complex over that of the antimalarial alone, over resistant strains may find the most likely explanation in the fact that at equivalent concentration, the complex would reach a quantity effective in DV than the antimalarial would do alone, because of its influx out of DV

[21]. The genetic determinism of *Plasmodium* chemoresistance to APQs is linked to the point mutations of the Pfcrt and PfMDR1 genes located on chromosomes 7 and 5 of *P. falciparum* respectively [22, 23].

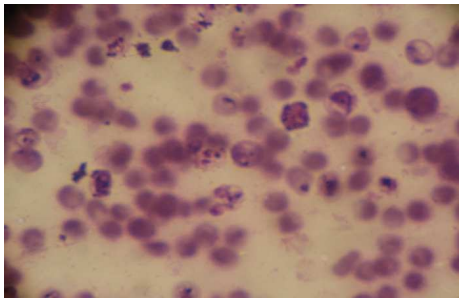

Figure 1. Thin smear of Erythrocytes of infected mice.

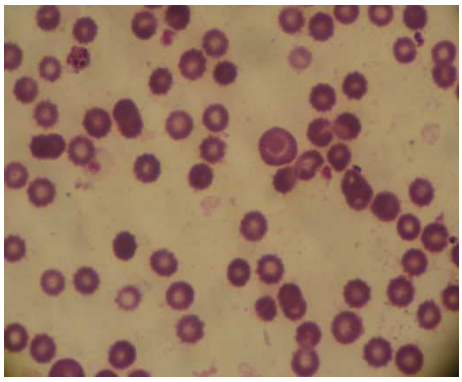

Figure 2. Thin smear Erythrocytes of infected mice after treatment.

Table 4 below gives the average weights of mice before and after the exposure to the hemin-amodiaquin complex.

Table 4. Average weights of Balb/c mice before and after exposure to the hemin-amodiaquin complex (1.6 mM/Kg) 0.1M Tris buffer, water-propylene glycol mixture 30%.

Group	Weight (gr) at D_0	Weight (g) at D_{14}	ΔP (%)
T_{01}	20,7±0,8	23,4±1,1	13
T_1	22,0±0,7	23,6±1,8	7.3
T_2	21,6±1,3	23,4±1,1	8.3

(Legend: T_{01} and T_{02}: Control Groups 1 and 2; T 1: Test group; ΔP (%): Weight gain).

As it can be observed, there is a slight weight gain in each group.

This gain is of the same magnitude in both the test group (T_1), i.e. having been exposed to the hemin-amodiaquin complex, and in the control groups (T_{01} and T_{02}) not exposed. The application of Student's statistics shows that there is no significant difference (p. 05: both for T_1 with T_{01} and for T_1 with T_{02}) between these gains attesting to the used concentration, the complex is not toxic. It should be noted that amodiaquin or chloro-7 (diethylaminomethylmethyl-3' hydroxy-4'anilino)-4-quinoline has recently been reported as highly cytotoxic because its biotransformation by polynuclear leukocytes would lead to the formation of a toxic iminoquinonic derivative [24] and justifies its choice in this pilot study.

The antiplasmodial activity of different complexes with their complexation constants (thermodynamic stability) is presented in the table below.

Table 5. Results of correlation assay between complexation constant K (at 37°C and pH 7.4) and CI_{50} of tested drugs.

Class	Complex	IC_{50} (nmol/L)	K ($10^4 M^{-1}$) [25]
I.	H-Q	38±14	2,8±0,90
	H-QD	65±2	42,9±0,50
	H-MQ	54±5	0,21±05
II.	H-CQ	102±13	4,54±0,74
	H-AQ	74±9	1,6±0,40

(Legend: I: Hemin-carbinol-4-quinoleins complex; II: Hemin-amino-4-quinoines complex).

It is found that within the class of amino-4-quinoleins, the more the complex is stable (high K value), the less is its parasitological activity. Indeed, it is noted that the hemin-chloroquine complex is less active than the hemin-amodiaquine complex while their stabilities go in the opposite direction. On the other hand, in the carbinol-4-quinolein class, this correlation appears less clear. It is now only for the hemin-quinidine and hemin-mefloquine complexes. The hemin-quinine complex does not obey it. Considering all the complexes, the correlation also seems less obvious. Only three out of five complexes respond to this correlation. These are the complexes of hemin with chloroquine, amodiaquine and mefloquine. The absence of a clear correlation between the stability and antimalarial activity of the complexes is likely due to the large difference in sensitivities of the plasmodial strains used.

As observed, the correlation is, in fact, observed only in the case of complexes tested on strains of sensitivities quite close. It should be noted that if this reverse correlation between thermodynamic stability and the antimalarial activity of complexes were to be confirmed, it would indicate that the activity of the APQs would not only imply their ability to complex hemin bio-cristallized to put it back into solution (inhibition of the formation of hemozoin or hematine) but also on the ability of the complex formed to dissociate itself.

4. Conclusion and Suggestions

In this study, we compared the parasitological activity of APQs with those of the complexes they form with hemin, on the one hand, and evaluated the *in vivo* antimalarial activity of the hemin-amodiaquine complex and its toxicity on Balb mice. /c. Finally, we sought to determine a possible correlation between the biological activity of complexes and their thermodynamic stabilities.

The results of the *in vitro* plasmodial test indicate that all of the complexes tested are biologically more active than the corresponding APQs alone ($p<0.05$). This superiority is also demonstrated *in vivo* with the hemin-amodiaquine complex compared to chloroquine. In addition, the rate of inhibition of plasmodies by hemin is very low supporting thus the hypothesis which attributes the antiplasmodial activity of APQs to the complexes they form with hemine in the VD of the *P. falciparum*. The test to assess the toxicity of the hemin-amodiaquine complex indicates that at the concentration of the work, the latter is not toxic.

This result should then be confirmed with the cytotoxicity test to determine the concentration in complex that inhibits cell growth by 50% (IC_{50}) and determine the toxic minimal dose (TMD) *in vivo*. At last, there is no clear correlation between the antimalarial activity of all the complexes studied and their thermodynamic stability. It can still be observed that the more stable the complex is, the less its antimalarial activity. The overall results obtained in this study pave the way for further research on hemin complexes of APQs as a "new active principle." To this end, it is therefore desirable that the plasmodial test be carried on standardized parasitic strains using the isotopic test. It would be interesting to expand this study to other APQs derivatives and corresponding complexes in order to confirm the thermodynamic-biological activity correlation of complexes by determining an area of temperature, pH, ionic strength or the reaction environment to which the complexation constant would respect the order of parasitological activity.

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