

Original Research Article**Influence of substituents nature on the antiplasmodial and cytotoxic activities of 5-chloro-3,4-dihydro-1*H*-pyrano[3,4-b]-1,10-phenanthrolinone as antimalarial agent** **Abstract**

Purpose: Malaria is one of the most common parasitic disease in tropical and subtropical regions. In human four species of *Plasmodium* are responsible for this disease. The most serious infections among these species are caused by *P. falciparum*. Because of the widespread and ever increasing resistance against existing malarial drugs, there is increasing need for new therapeutic agents against malaria.

Methods: A series of 7 substituents were used to substitute the chlorine on the 5 position in the 5-chloro-3,4-dihydro-1*H*-pyrano[3,4-b]-1,10-phenanthrolinone ring. *In vitro* antiplasmodial activities were evaluated on chloroquine-sensitive and resistant strains of *P. falciparum*.

Results: The results showed that compounds **7** and **8** possessing N,N-diethylamino side chain had the best antiplasmodial activities. In addition, the cytotoxic activities were evaluated on HeLa cells and compound **8** was the less cytotoxic of all studied compounds.

Conclusion: The interesting profiles of compound **8** should be due to its lipophilic and basic characteristics.

Keywords: malaria disease, *Plasmodium falciparum*, 1,10-phenanthrolinone, antimalarial activity

25 Introduction

26 Malaria is one of the tropical parasitic diseases with the highest global mortality. According
 27 to the world health Organization, 6.2 million fever malaria deaths were occurred globally in
 28 the world between 2001 and 2015, and 438000 deaths were registered in 2015 [1]. Over 81%
 29 of case and 91% of the deaths in 2015 were in Africa, and the majority of the remaining
 30 being in India, Southeast Asia and South America. Malaria is a disease caused by parasite
 31 from the genus *Plasmodium* and is transmitted to a human host when the vector, an infected
 32 female

33 *Anopheles* mosquitos feeds on a human host. *P. falciparum* and *P. vivax* are responsible for
 34 most of human malarial infection. For many African tropical countries, this disease is one of
 35 the major public health problem [2]. In additional, malarial burden is exacerbated with the
 36 spread of drug resistance of *P. falciparum* to antimalarial drugs and of *Anopheles* to
 37 insecticides [3]. Fighting against *Plasmodium* drug resistance requires the development of
 38 new candidates as potential efficient antimalarial drugs. In previous studies, consisting to
 39 evaluate the diaza analogs of phenanthrene for their antimalarial activities against both
 40 chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains, we found that 4-chloro-
 41 3-(2-chloroethyl)-2-methyl-1,10-phenanthroline **1** (**figure 1**) had the most efficient activity
 42 [4-5]. To obtain more informations on structure-activities relationships of such compound,
 43 we used an efficient oxidation route by using 1.5 equivalent of SeO₂ in pyridine at 50 °C to
 44 get the optimal yield of 5-chloro-3,4-dihydropyrano[3,4-*b*]-1,10-phenanthrolinone **2** [6].
 45 When the reaction conditions were enhanced to 3 equivalent of SeO₂ at 115 °C, two
 46 compounds were formed, besides compound **2**, compound **3** which can be in two tautomeric
 47 forms **3a** and **3b** (**figure 2**). Access to product **2** has been valued by a pharmacomodulation
 48 which consist to substitute the chlorine carried by the pyridine ring by various substituents
 49 having amino groups. Indeed, it has been shown that the introduction of amino groups having

units of type morpholine, alkyldiamine, piperazine, etc., brings better antiplasmodial activities to other family of molecules. Other authors have shown that some fluoroquinolones such as ciprofloxacin, perfloxacin and norfloxacin with the piperazin or methylpiperazin motif in their structure have promising antiplasmodial activity *in vitro* on chloroquine-resistant *P. falciparum* strains [7-8]. Other patented series of molecules type of pyrrolidino-alkylamine with the pattern N,N-diethylaminopropyl such as (CDRI 87/209) and (WR268954) have antiplasmodial activities on chloroquine-resistant strains [9]. From these observations, it was an interesting challenge to study on one hand the reactivity of compound **2** and its analogs obtained by substitution of the chloride in position 5 with different amino group mentioned in (figure 3) and on the other hand to compare their antiplasmodial activity on both chloroquine-sensitive and -resistant strains and their cytotoxicity on *HeLa* cells.



Figure 1: structure of compounds **1** and **2**

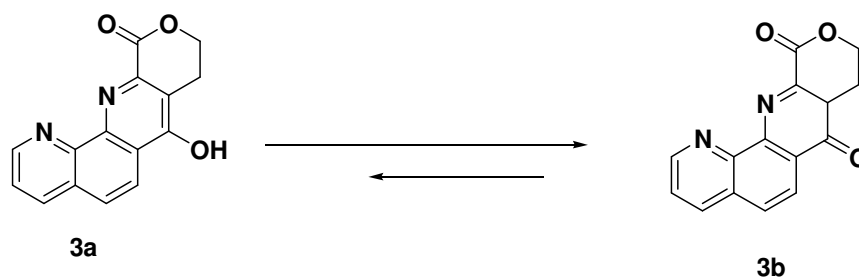


Figure 2: structure of tautomeric forms of **3a** and **3b**

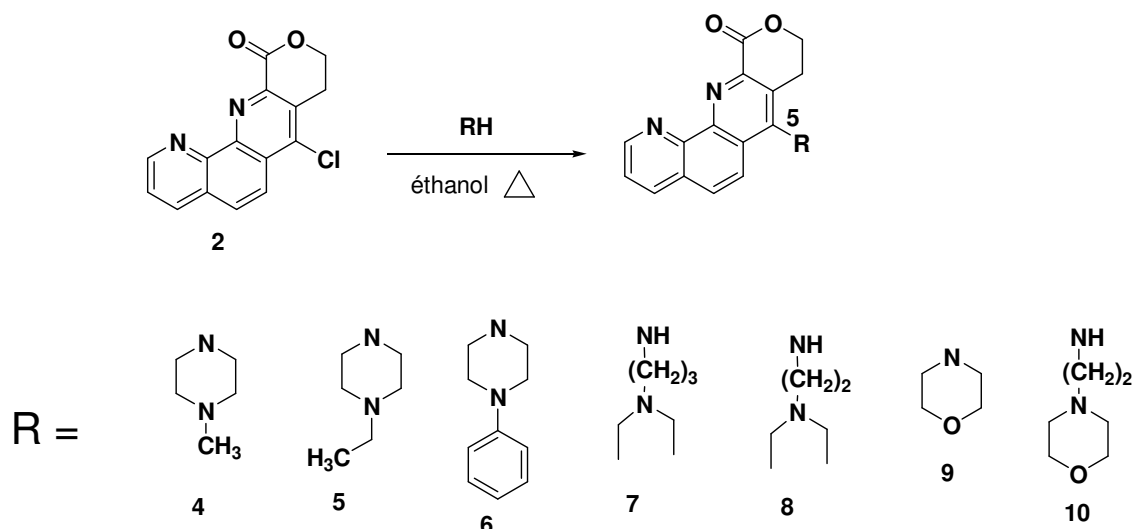


Figure 3: The amino groups used to substitute the chlorine of compound 2

Material and methods

General Experimental.

All reagents were used as supplied commercially; the solvents were freshly distilled before use. ^1H and ^{13}C NMR spectra were measured on a Bruker 300 MHz spectrometer with CDCl_3 , MeOD, DMSO as deuterium solvents. All chemical shifts were recorded in δ (ppm) relative to tetramethylsilane. Melting points were measured with a Stuart Scientific, melting point apparatus SMP3. The infrared spectra were taken in KBr disks using an ABB Bomen MB series spectrometer with spectra resolution of 2 cm^{-1} . The mass spectra analyses were recorded on a finnigan DSQ spectrometer operating at 70 electrons volt. Aluminium-backed silica plates with UV indicator and silica gel 37-70 μm , 200-400 mesh (SDS-13124 Peypin, France) were used in analytical thin-layer chromatography (TLC) and silica gel chromatography respectively. The spots were visualized by UV lamp (254-365 nm) or by using the iodine.

87 **Obtention of the 5-chloro-3,4-dihydro-1*H*-pyrano[3,4-*b*]-1,10-phenanthrolinone (2)**

88 To a solution of 4-chloro-3-(2-chloroethyl)-2-méthyl-1,10-phenanthroline **1** (0.20 g, 0.69
89 mmole) in anhydrous pyridine (5 ml) was added SeO₂ (0.2g, 0.69 mmole). The solution was
90 refluxed for 3 hours. The hot solution was filtered and the solvent was evaporated to give a
91 residue which was after addition of a little amount of water, extracted with chloroform. The
92 organic layer was dried over Na₂SO₄ and evaporated under vacuum. The crude material was
93 purified by column chromatography through silica gel eluted by dichloromethane/methanol
94 (95/5) to give lactone compound (**2**).

96 **General methods for preparation of compounds 4 to 9.**

97 To a stirred solution of 5-chloro-3,4-dihydropyrano[3,4-*b*]1,10-phenanthrolinone **2** (0.11g,
98 0.39 mmole) in distilled absolute ethanol (5 ml), was added the amine compound
99 (substituent). The solution was refluxed for 3 hours. Back at room temperature, the solution
100 was evaporated under vacuum pressure. The crude mixture was suspended in 50 ml of water
101 and pH of the solution was adjusted to pH= 8. The aqueous layer was extracted with
102 dichloromethane (3x50 ml), then the organic layer dried over Na₂SO₄, filtered and
103 evaporated. The obtained solid was precipitated in cold ether or was purified by flash
104 chromatography eluted with dichloromethane-methanol (9/1).

105 **Antiplasmodial and cytotoxic activities evaluation**

106 *In vitro* antimalarial activity was evaluated on 3 references *P. falciparum* strains. FcB₁-
107 Colombia and FcM₂₉-Cameroun were considered to as chloroquine resistant and F₃₂-Tanzania
108 as chloroquine sensitive. *P. falciparum* was cultured by using the method described by [10]
109 with modifications [11]. Cultures were synchronized by 5% of D-sorbitol (Sigma, France)
110 which lysed the old stage parasites. The evaluation was performed on 96 well plates (TPP,
111 Suisse) which were filled with 100 ml of *P. falciparum* culture (parasitemia =1.5%, and

hematocrit =1.5%) to which was add 100 ml of culture medium containing the compounds to be tested at growing dilutions. After 24 h incubation at 37 °C with 5% CO₂, 7400 Bq of [³H]-hypoxanthine (Perkin Elmer) was added in each well. The plates were returned in incubator for 24 h more. After nucleic acids collection on a nylon filter (Perkin-Elmer) [³H] hypoxanthine incorporation was measured with a β counter (1450-Microbeta Trilux-Perkin Elmer). Results were then analysed for the determination of the 50% inhibitory concentration (IC₅₀), compared to control without drugs (100% incorporation) and red blood cells without parasites (0% incorporation).

Cytotoxicity of the drugs was estimated on *HeLa* cells which were cultured in similar conditions than for *P. falciparum* except for the 5% human serum which was replaced by 5% fetal calf serum (Boehringer) as described elsewhere [12]. After addition of drugs at various concentrations, cell growth was estimated by [³H] hypoxanthine incorporation after 48 h incubation.

Computational methods

Structure of heme was extracted from the 5sw7.pdb file obtained from RCSB protein databank (<http://www.rcsb.org>). Structure of compounds (**8**, **9**) were built in HyperChem Release 8.0.5 pro for Windows (Hypercube Inc. Gainesville, Florida). The geometries were optimized using the semi-empirical AM1 programme in singly-excited configuration interaction. (RHF [Restricted Hartree-Fock], charge 0, spin multiplicity 1, lowest state, orbital criterion, five occupied and five unoccupied orbitals.) to an rms (root mean square) gradient of 0.01 in vacuo (Polak-Ribière method). The binding mode of **8** and **9** to heme was analyzed by use of ArgusLab 4.0 using a bounding box of 25x25x25 Å, using grid (0.4 Å), Argusdock engine, flexible ligand, high precision, and no augmented torsions. The energetically most favorable binding pose of **8** (binding energy = -8.0 kcal/mol) and **9** (binding energy = -7.28 kcal/mol) with heme, were then subjected to molecular dynamic

using Hyperchem 8.05. The system was first optimized in MM+ to an rms gradient of 0.5. Then a molecular dynamics program was run for 1 ps, with 0.001 ps steps, relaxation time 0.1 ps, to a simulation temperature of 300 K. This was followed by MM+ geometry optimization to an rms gradient of 0.2. The molecular dynamics run was repeated and a further MM+ protocol was carried out to a gradient of rms 0.01.

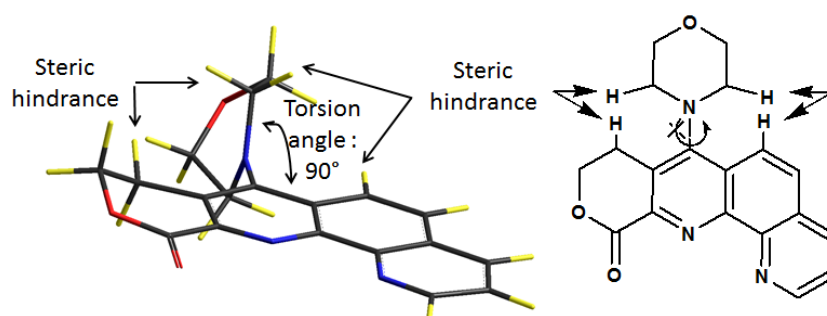


Figure 4: restricted conformation of compound 8

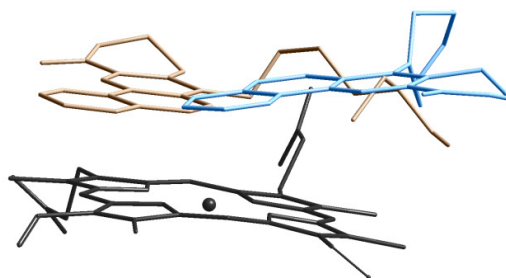


Figure 5: superimposition of the best docked poses of 8 (brown) and 9 (blue) with heme (black)

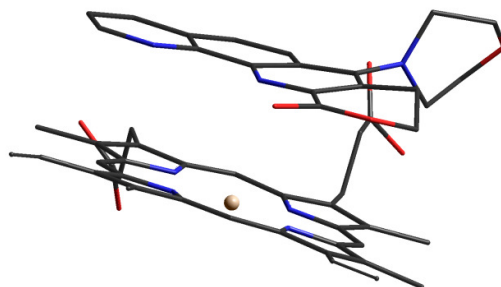


Figure 6: refined interactions for 9 with heme (molecular dynamics)

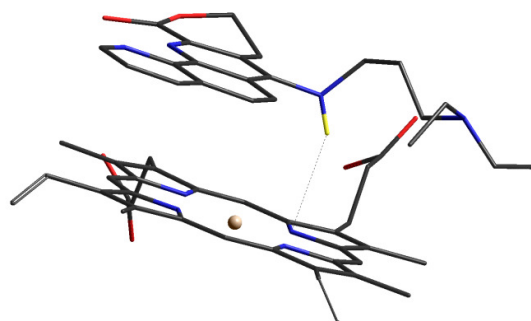


Figure 7: refined interactions of 8 with heme (molecular dynamics) showing H-bonding

Results and discussion

All the synthesized compounds (**1** to **10**) were screened for their antiplasmodial activities on the three strains of *P. falciparum* strains (chloroquine- resistant FcB₁ and FcM₂₉ and chloroquine-sensitive F₃₂). Five molecules were screened for their cytotoxicity toward *HeLa* human cell line. 

169 **Table 1.** *In vitro* antiplasmodial and cytotoxicity activities, IC₅₀ in μ M

	FcB ₁ ^b	FcM ₂₉ ^b	F ₃₂ ^b	HeLa
Compounds ^a	48 h ^c	48 h ^c	48 h ^c	48 h ^c
2	98.59 $\pm$ 08	61.97 $\pm$ 07	68.66 $\pm$ 2.13	NT
3a	11.27 $\pm$ 1.70	12.03 $\pm$ 1.06	8.45 $\pm$ 1.32	112 $\pm$ 3.50
4	83.33 $\pm$ 070	91 $\pm$ 0.30	89.08 $\pm$ 4.06	NT
5	30.38 $\pm$ 0.19	71.82 $\pm$ 0.9	88.39 $\pm$ 00	NT
6	87.80 $\pm$ 10	6.58 $\pm$ 1.30	46.34 $\pm$ 2.44	21.95 $\pm$ 1.63
7	18.51 $\pm$ 1.50	6.87 $\pm$ 1.50	11.37 $\pm$ 2.05	92.59 $\pm$ 3.51
8	11 $\pm$ 2.13	6.86 $\pm$ 1.32	6.86 $\pm$ 1.13	247 $\pm$ 26
9	101 $\pm$ 0.01	78.35 $\pm$ 2.18	95.52 $\pm$ 00	NT
10	74.07 $\pm$ 09	52 $\pm$ 0.02	56.87 $\pm$ 2.61	92.59 $\pm$ 9.33

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171 ^a= compounds, ^b = parasites ; ^c = incubation time ; **NT** = non tested

172

173 **Table 2: Selectivity index measured for 4 compounds**

Compounds	FcB ₁ ^b	FcM ₂₉ ^b	F ₃₂ ^b
3a	9.93	9.31	13,26
7	5	13.47	8.14
8	22	36	36
10	1.25	1.78	1.62

174

175 **IC₅₀** = concentration corresponding to 50% growth inhibition of the parasite

176 Selectivity index (**SI**) = IC₅₀ values of cytotoxic activity/IC₅₀ values of antimalarial activity

Antimalarial potential of such compound **2** possessing a 1,10-phenanthroline basic framework depends essentially on the nature of the substituent used to substitute the chlorine on the position 5. Indeed, our results showed that the chlorine in the 5 position caused a loss of antiparasmodial activity. Compound **3**, where chlorine was replaced by a ketone function, had antiparasmodial activities ranging from 5 to 9 times greater than that of compound **2** on the three *P. falciparum* strains. Compared to compound **9** where the substituent was morpholine, compound **2** presented the most interesting antiparasmodial activities. In compound **10**, the presence of N-ethyl chain led to a significant improvement of the antiparasmodial activity on the 3 tested strains. This chain increased the lipophilicity of the molecule which could facilitate its penetration and availability in the cytoplasm of the parasite. Contribution of piperazine substituents (compounds **4**, **5** and **6**) on the activity of compound **2** was not too significant with the exception of compound **6** where activity on FcM₂₉ was almost 10 times greater than compound **2**. The presence of the lipophilic aromatic ring might explain this difference in antiparasmodial activity. The substituents of diaminoalkyl nature (compounds **7** and **8**) were the best for their contribution in enhancing antiparasmodial activity of compound **2**. In fact, these two compounds had more interesting activities than those of the compound **2** on the three *P. falciparum* studied strains.

For its amino acids providing, the parasite in the host erythrocyte degrade the hemoglobin which release amino acids for its maturation and toxic heme which accumulates in the food vacuole. Lacking heme oxygenase activity to catalyze the porphyrin moiety, parasite polymerize the highly free reactive heme into a metabolically inert crystalline material called hemozoin. This substance is a polymer of undefined length, in which the iron of one heme is coordinated to the side chain propionate carboxylates of the next heme [13]. The quinoline antimalarial drugs such as chloroquine and quinidine inhibit the polymerization of heme to hemozoin which is toxic for the *Plasmodium*.

Chloroquine has been demonstrated to bind to ferriprotoporphyrine (Figure 4) with high affinity *in vitro* and has been suggested that the drug can sequester heme interfering with the formation of the inert pigment and the complex can lyse the parasite membrane [14]. Pashka and coworkers have demonstrated that the mode of action of this compound is based on its association with heme or with different polymers of this one by noncovalent bonds [15]. Egan and colleagues have shown that the complexation between chloroquine and heme, is guided by H-bonding as well as π -stacking interactions between the aminoquinoline and heme [16]. In the light of these informations, studying the mode of interaction of the most active compound **8** and the less active one (**9**) should afford fundamental informations about their mode of action. First, it is obvious to note that compound **9** is conformationnaly restrained with the morpholine ring perpendicular to the quinoline framework and that the nitrogen in the 5 position cannot be implicated in H-bonding as an H-donnor (figure 4 and figure 5). These structural specificities greatly oriented the mode of interaction of compound **9** with heme when compared to compound **8**. Although the binding energies of **8** and **9** to heme are quite similar, we can note that the presence of a blocked morpholine ring in **9** result in reduced P-P stacking interactions between compound **9** and heme (figure 6), and that no H-bonding between the two molecules can be founded in contrast with compound **8** which can interact favorably (P-P stacking and H-bond) with heme (figure 7).

For the cytotoxicity measurement, compounds **3a**, **7**, **8** and **10** are least toxic activity on HeLa cell line with IC₅₀ respectively 112, 95, 247 and 92 μ M. Indeed, most the IC₅₀ is greater, lower the compound is potentially toxic. Thereby, compound **8** seems to be less cytotoxic on the studied cell line. However, the selectivity index fail to pass to the *in vivo* test because according to Long-Ze, it's must be greater than or equal to 100  *in vivo* test is permitted [17].

Conclusion

Drug resistance of parasites and anopheles remains a major problem in the malaria burden. At present, the chemically best solution to eradicate definitely this disease is the establishment of new antimalarial carrier which is exempt of any resistance. The 1,10-phenanthroline skeleton was synthesized and studied for its potential new antimalarial support. Its pharmacomodulation led to several compounds among which compound **8** provides an interesting lead in our search for the activity improvement of compound **2**. This compound should be used for future pharmacomodulation conducting particularly to open the lactone ring in basic medium.

Consent and Ethical approval, are not applicable in this study



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