

Influence of substituents 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

Background: Malaria is one of the most common parasitic disease in tropical and subtropical regions. *Plasmodium falciparum*, one of the causative agent of malaria is resistant to many type of antimalarial drugs. This study present *in vitro* assessment of the antiplasmodial activity of substituted phénanthrolinone compounds.

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 least cytotoxic of all studied compounds.

Conclusion: The synthesis and antimalarial activity of substituents of compound **2** were carried out. This study has shown that compounds with basic and lipophilic substituents exhibited the best antiplasmodial activity.

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, it was estimated 212 million cases of malaria occurs
28 worldwide and 429 000 deaths were registered in 2015 [1]. Over 81% of cases and 91% of the
29 deaths in 2015 were in Africa, and the majority of the remaining being in India, Southeast
30 Asia and South America. Malaria is a disease caused by parasites from the genus
31 *Plasmodium* and is transmitted to a human host when the vector, an infected female
32 *Anopheles* mosquito feeds on a human host. *P. falciparum* and *P. vivax* are responsible for
33 most of human malarial infection. For many African tropical countries, this disease is one of
34 the major public health problems [2]. In addition, malarial burden is exacerbated with the
35 spread of drug resistance of *P. falciparum* to antimalarial drugs and of *Anopheles* to
36 insecticides [3]. Fighting against *Plasmodium* drug resistance requires the development of
37 new candidates as potential efficient antimalarial drugs. In our previous studies, antimalarial
38 activity of diaza analogues of phenanthrene were evaluated for their antimalarial activities
39 against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains. These
40 studies revealed that 4-chloro-3-(2-chloroethyl)-2-methyl-1,10-phenanthroline **1** (figure 1)
41 had the most efficient activity [4-5]. To obtain more information on structure-activity
42 relationship of such compound, an efficient oxidation route involving 1.5 equivalent of SeO₂
43 in pyridine at 50 °C was adopted to get the optimal yield of 5-chloro-3,4-dihydropyrano[3,4-
44 *b*]-1,10-phenanthrolinone **2** [6]. When the reaction conditions were modified to 3 equivalent
45 of SeO₂ at 115 °C, two compounds were formed including compound **2**. The other compound
46 formed, compound **3** appears in two tautomeric forms **3a** and **3b** (figure 2). Access to product
47 **2** has been valued by a pharmacomodulation. The chlorine of the pyridine ring was substituted
48 by various substituents having amino groups. Indeed, it has been shown that the introduction
49 of amino groups having units of type morpholine, alkyldiamine, piperazine, etc., brings better

antiplasmodial activities to other family of molecules[7-9]. 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* against chloroquino-resistant *P. falciparum* strains [10-11]. 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 against chloroquino-resistant strains [12]. From these observations, the challenge was thrown to study on one hand the reactivity of compound **2** and its analogues 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 against both chloroquine-sensitive and resistant strains and their cytotoxicity against *HeLa* cells.



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

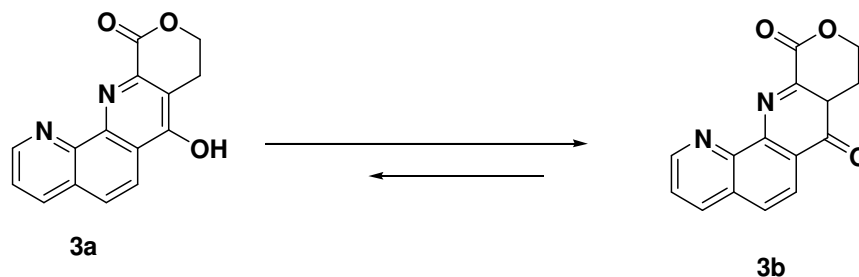


Figure 2: Structure of tautomeric forms of **3a** and **3b**

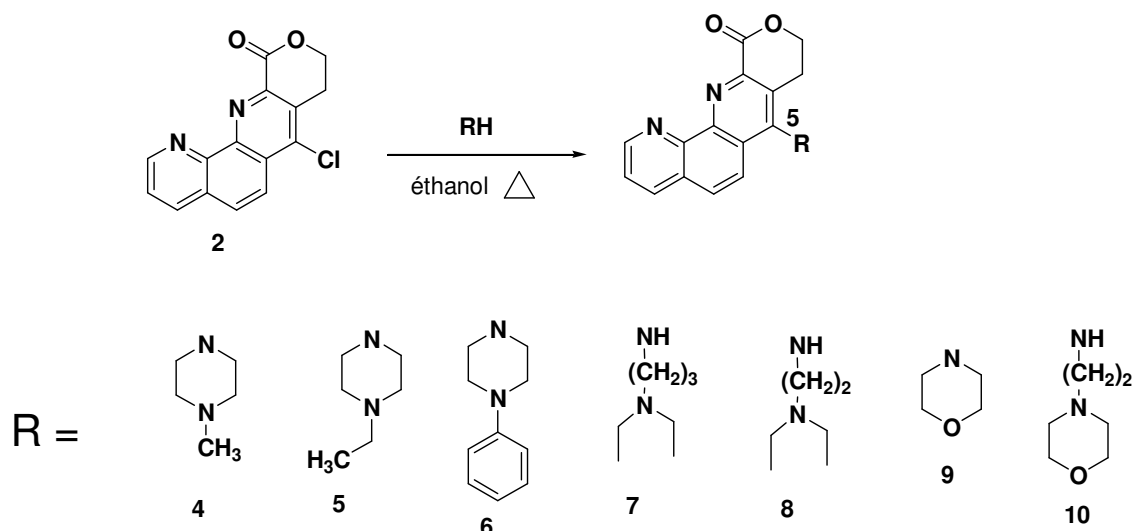


Figure 3: The amino groups used to substitute the chlorine of compound 2

Material and methods

General Experimental.

All reagents were 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 deuterated 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.

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

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

General methods for preparation of compounds 4 to 9.

To a stirred solution of 5-chloro-3,4-dihydropyrano[3,4-*b*]1,10-phenanthrolinone **2** (0.11g, 0.39 mmole) in distilled absolute ethanol (5 ml), the amine compound (substituent) was added. The solution was refluxed for 3 hours. Cooled down at room temperature, the solution was evaporated under vacuum pressure. The crude mixture was suspended in 50 ml of water and pH of the solution was adjusted with Na₂SO₄ solution to pH= 8. The mixture was extracted with dichloromethane (3x50 ml), then the organic layer dried over Na₂SO₄, filtered and evaporated. The remaining oil was treated with ether cooled and the precipitate was filtered. When the compound did not precipitate, the purification was carried out by flash chromatography with dichloromethane-methanol as eluent.

Antiplasmodial and cytotoxic activities evaluation

In vitro antimalarial activity was evaluated against 3 references *P. falciparum* strains. FcB₁-Colombia and FcM₂₉-Cameroun are chloroquine resistant and F₃₂-Tanzania is chloroquine sensitive. *P. falciparum* was cultured in schizont stage of development by using the method described by Trager et al[13] with modifications[14]. Cultures were synchronized by 5% of

D-sorbitol (Sigma, France) which lysed the old stage parasites. The evaluation was performed in 96 well plates (TPP, Suisse) which were filled with 100 ml of *P. falciparum* culture (parasitemia =1.5%, and hematocrit =1.5%) to which was added 100 ml of culture medium containing the compounds to be tested at different dilutions. After 24 h incubation at 37 °C with 5% CO₂, 7400 Bq of [³H]-hypoxanthine (Perkin Elmer) was added to each well. The plates were further incubated for additional 24 hours. 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) and compared to controls by using the dose response curves of excel software.

Cytotoxicity of the drugs was estimated on *HeLa* cells which were cultured in similar conditions as for *P. falciparum* except for the 5% human serum which was replaced by 5% fetal calf serum (Boehringer) as described elsewhere [15]. After addition of drugs at various concentrations, cell growth was estimated by [³H] hypoxanthine incorporation after 48 hours 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 invacuo (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 favourable 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.

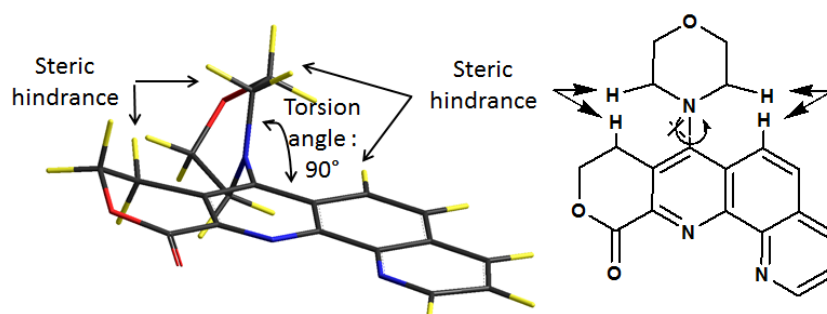


Figure 4: Restricted conformation of compound 8

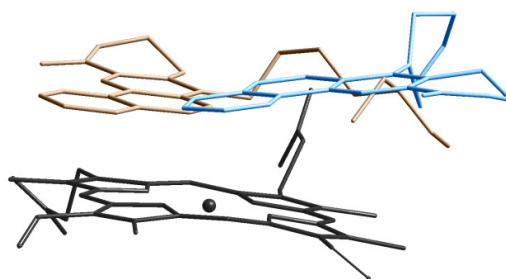


Figure 5: Superimposition of the best docked poses of 8 (brown) and 9 (blue) with heme (black)

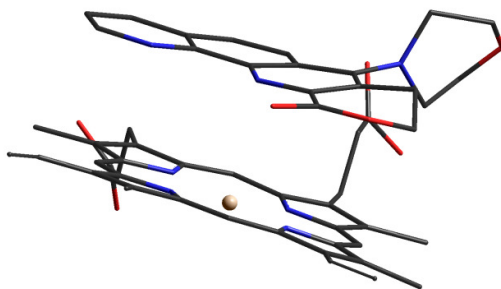


Figure 6: Refined interactions for 9 with heme (molecular dynamics)

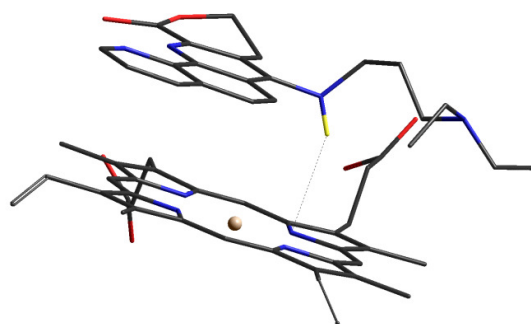


Figure 7: Refined interactions of 8 with heme (molecular dynamics) showing H-bonding

Results and discussion

All the synthesized compounds (**1** to **10**) were screened for their antiparasmodial activities against 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.

Table 1. *In vitro* antiparasmodial 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 ± 1.70	12.03 ± 1.06	8.45 ± 1.32	112 ± 3.50
4	83.33 ± 0.70	91 ± 0.30	89.08 ± 4.06	NT
5	30.38 ± 0.19	71.82 ± 0.90	88.39 ± 2.70	NT
6	87.80 ± 1.00	6.58 ± 1.30	46.34 ± 2.44	21.95 ± 1.63
7	18.51 ± 1.50	6.87 ± 1.50	11.37 ± 2.05	92.59 ± 3.51
8	11 ± 2.13	6.86 ± 1.32	6.86 ± 1.13	247 ± 26.00
9	101 ± 0.01	78.35 ± 2.18	95.52 ± 4.23	NT
10	74.07 ± 0.90	52 ± 0.02	56.87 ± 2.61	92.59 ± 9.33

a = compounds, **b** = parasites ; **c** = incubation time ; **NT** = non tested ; n = 3

Table 2: Selectivity index measured for 4 compounds

Compounds	FcB ₁ ^b	FcM ₂₉ ^b	F32 ^b
3a	9.93	9.31	13.26
7	5.00	13.47	8.14
8	22.00	36.00	36.00
10	1.25	1.78	1.62

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

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

Antimalarial potential of 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 antiplasmodial activity. Compound **3**, where chlorine was replaced by a ketone group, had antiplasmodial activities ranging from 5 to 9 times greater than that of compound **2** against

176 the three *P. falciparum* strains. However when compared to compound **9** where the
177 substituent was morpholine, compound **2** showed a relatively higher antiplasmodial activity.
178 In compound **10**, the presence of N-ethyl chain led to a significant improvement of the
179 antiplasmodial activity on the 3 tested strains. This chain increased the lipophilicity of the
180 molecule which could facilitate its penetration and availability in the cytoplasm of the
181 parasite. Contribution of piperazine substituents (compounds **4**, **5** and **6**) on the activity of
182 compound **2** was not too significant with the exception of compound **6** where activity on
183 FcM₂₉ was almost 10 times greater than compound **2**. The presence of the lipophilic aromatic
184 ring might explain this difference in antiplasmodial activity. The substituents of diaminoalkyl
185 nature (compounds **7** and **8**) were the best for their contribution in enhancing antiplasmodial
186 activity of compound **2**. These two compounds have registered higher activities than
187 compound **2** against all three of the *P. falciparum* parasite.

188 To provide amino acids, the parasite in the host erythrocyte degrades the hemoglobin which
189 releases amino acids for its maturation and toxic heme which accumulates in the food
190 vacuole. Lacking heme oxygenase activity to catalyze the porphyrin moiety, the parasite
191 polymerizes the highly free reactive heme into a metabolically inert crystalline material
192 called hemozoin. This substance is a polymer of undefined length, in which the iron of one
193 heme is coordinated to the side chain propionate carboxylates of the next heme [16]. The
194 quinoline antimalarial drugs such as chloroquine and quinidine inhibit the polymerization of
195 heme to hemozoin, which is toxic for the *Plasmodium*.

196 Chloroquine has been demonstrated to bind to ferriprotoporphyrine with high affinity *in vitro*
197 and has been suggested that the drug can sequester heme interfering with the formation of the
198 inert pigment and the complex can lyse the parasite membrane [17]. Pashka and coworkers
199 (2004) have demonstrated that the mode of action of this compound is based on its
200 association with hemin or with different polymers of this one by noncovalent bonds [18].

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[19]. In the light of this information, studying the mode of interaction of the most active compound **8** and the less active one (**9**) should afford fundamental information about their mode of action. First, it is obvious to note that compound **9** is conformationally 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-donor (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** resulted 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 favourably (P-P stacking and H-bond) with heme (figure 7).

For the cytotoxicity measurement, compounds **3a**, **7**, **8** and **10** have least toxicity on HeLa cell line with IC₅₀ respectively being 112, 95, 247 and 92 μ M. Indeed, most of the IC₅₀ are greater, lower the compound is potentially toxic. Thereby, compound **8** seems to be less cytotoxic on the studied cell line. However, the selectivity index of this compound is very low (36.00) which does not allow it to be tested *in vivo*. According to Long-Ze, the selectivity index must be greater than or equal to 100 the *in vivo* test is permitted [20].

Conclusion

Drug resistance of parasites and anopheles remains a major problem in the malaria burden. At present, the best chemically solution to eradicate definitely this disease is establishment of new antimalarial drug which is exempt of any resistance. The 1,10-phenanthroline skeleton was synthesized and studied for its potential new antimalarial activity. Its

pharmacomodulation led to several compounds among which compound **8** showed higher activity as compared to the parent compound **2**. This compound should be used for future pharmacomodulation particularly to open the lactone ring in basic medium.

Consent and Ethical approval, are not applicable in this study

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