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Graphical Abstract

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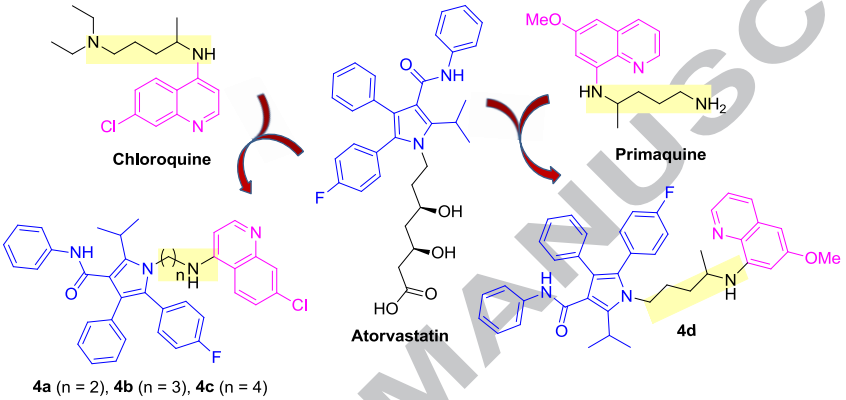

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ABSTRACT

Cerebral malaria is caused by *Plasmodium falciparum*. Atorvastatin (AVA) is a pentasubstituted pyrrole, which has been tested as an adjuvant in the treatment of cerebral malaria. Herein, a new class of hybrids of AVA and aminoquinolines (primaquine and chloroquine derivatives) has been synthesized. The quinolinic moiety was connected to the pentasubstituted pyrrole from AVA by a linker group $(CH_2)_{n=2-4}$ units. The activity of the compounds increased with the size of the carbons chain. Compound with n = 4 and 7-chloroquinolinyl has displayed better activity (IC_{50} = 0.40 µM) than chloroquine. The primaquine derivative showed IC_{50}=1.41 µM, being less toxic and more active than primaquine.

Malaria is a parasitic disease caused by protozoa of the *Plasmodium* genus and transmitted by *Anopheles* mosquitoes.[1] Drug resistance, documented in the species *P. falciparum*, and *P. vivax*, has been one of the main obstacles in the fight against malaria. In 2013, 584,000 people died due to cerebral malaria, the most severe manifestation that is caused by *P. falciparum*.[2] Of these deaths, 78% were among African children under five years old.[2] It is recommended that patients diagnosed with cerebral malaria should be treated with quinine, quinidine and one of the artemisinin derivatives, preferably by intravenous artesunate.[3]

Pyrrole is a simple aromatic heterocycle commonly found in nature that shows varied biological activity.[4,5] Substituted pyrroles have shown antiplasmodial activity[6-11] (Fig. 1). An isoquine analog containing tetrasubstituted pyrrole **1** was shown *in vitro* to have IC_{50} = 42.5 nM.[6] The trisubstituted pyrrole **2** inhibited the entry of *Plasmodium* sporozoites with *in vitro* and *in vivo* IC_{50} values of 10 µM and < 100 nM, respectively,[7,8] blocked the development of erythrocytic schizonts *in vitro* and was also partially effective against the erythrocyte stages of *P. berghei in vivo*.[9] The disubstituted pyrrole **3** showed high activity *in vitro* against *P. falciparum* (IC_{50} = 4.6 nM),[10,11] which was probably potentialized by a moiety of artemisinin derivative in its chemical structure.

Atorvastatin (**AVA**, Fig. 1) is one of the leading pyrrole drugs. Employed in hypercholesterolemia treatment for its inhibition of the HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) enzyme, AVA has been shown to be active against *P. falciparum*[12] and to promote a synergistic effect with dihydroartemisinin (DHA) activity.[13,14]

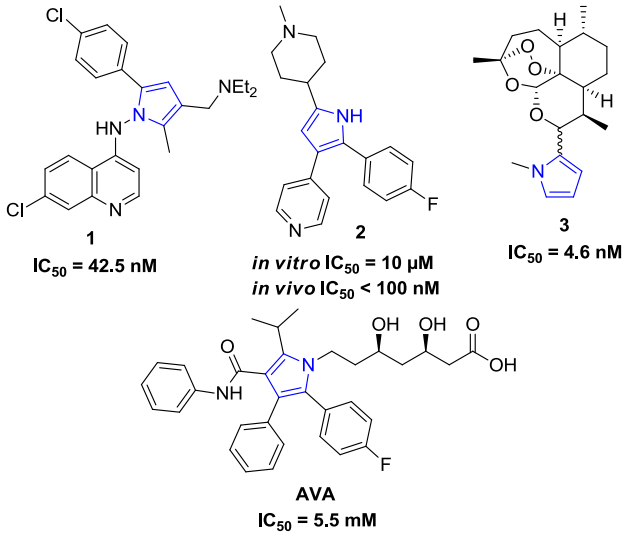


Figure 1: Known pyrroles with antiplasmodial activity.

AVA has shown significant pleiotropic effects such as neuroprotective and anti-inflammatory activities.[15] AVA is considered to be an outstanding adjuvant in the treatment of cerebral malaria[14,16-21], having been shown to inhibit the increased plasma and cerebrospinal fluid levels of interferon gamma

induced protein 10 (CXCL10), which is a chemokine secreted by several cell types in response to IFN-γ in fatal human cerebral malaria.[16-18] Unfortunately, no clinical trials in cerebral malaria using AVA as adjuvant of an artemisinin-based combination therapy have been yet performed. Studies have demonstrated an absence of cross-resistance *in vitro* between AVA and antimalarials, suggesting different modes of action and indicating that AVA can be potent against malaria.[12-13, 19-22]

Studies using *in silico* and *in vitro* tests with atorvastatin were carried out with lactate dehydrogenase of *P. falciparum* (*Pf*LDH), a crucial enzyme for *Plasmodium*'s anaerobic lifestyle and survival during the parasite erythrocytic cycle in the vertebrate host. Atorvastatin inhibited the enzyme function and is likely to act as therapeutic agent targeting parasite.[23]

Combination therapies are recommended by the WHO because the use of monotherapy for decades contributed to the emergence of strains resistant to excellent medicines such as cloroquine and mefloquine.[24] Currently, the molecular hybridization of two pharmacophoric groups in the same molecule has become an important tool in medicinal chemistry[25-27] and is the main goal of the present work. Hybridizations may improve pharmacological drug profiles by dual or new mechanisms of action, making it possible to overcome the parasite resistance mechanisms.[25-27]

In our previous search for new anti-malaria drugs, the effectiveness of hybrid salts of mefloquine and artesunate,[28] primaquine and artesunate,[29] 2-(trifluoromethyl)[1,2,4]triazolo[1,5-*a*]pyrimidine,[30] 1*H*-1,2,3-triazole-quinoline,[31] and quinoline-sulfonamide[32] hybrids against *P. falciparum* was demonstrated.

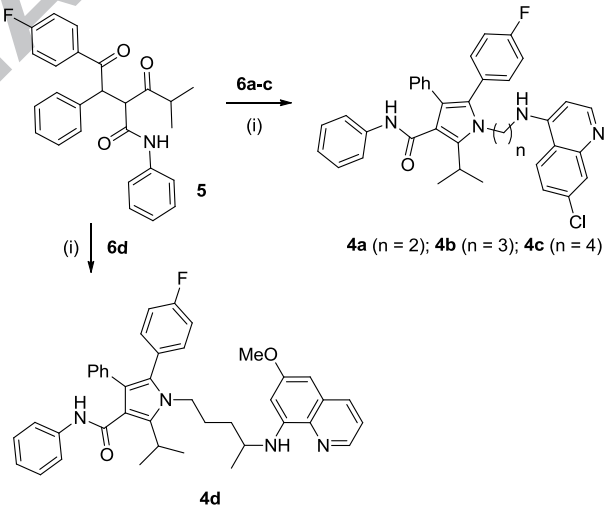
Chloroquine is an inexpensive and safe 4-aminoquinoline that has been a standard in malaria chemotherapy for decades because it is highly efficient against blood schizonts of all *Plasmodium* species. Malaria parasites grow within the red blood cells of host, and is believed that the efficacy of chloroquine is due its ability of blocking the hematin detoxification.[33] Its use as a single drug has been abandoned in the case of *P. falciparum* due to the emergence of resistant strains.[33] Primaquine is an 8-aminoquinoline and the only drug available to cure the late relapses caused by the *P. vivax* and *P. ovale* liver stages termed hypnozoites. However primaquine causes hemolysis in patients with G6PD deficiency. The search for new analogues or substitutes for chloroquine or primaquine has been a challenge.[34]

Using the molecular hybridization tool, the objective of this study was to synthesize and test a new class of hybrid derivatives with AVA and aminoquinolines in the structure. AVA was chosen based on its proven activity against malaria parasites and also because it is a highly substituted pyrrolic compound (Fig. 2).

The aminoquinoline moiety was included because it is present in chloroquine and in the primaquine, as 7-chloroquinolin-4-yl and 6-methoxyquinolin-8-yl parts, respectively. In the proposed hybrids, the quinoline moiety was connected to the pentasubstituted pyrrole by a linker group containing 2–4 CH_2 units (Fig. 2).

The synthesis of the new pyrrole-atorvastatin derivatives **4a-d** (Scheme 1) was achieved through the Paal-Knorr reaction,[35] between 1,4-diketone **5** and aminoquinolines **6a-d** at 80-100°C in 26-91 hours,[35,36] and the process was not optimized. Toluene can be used as the solvent, but a 1:1 mixture of tetrahydrofuran and cyclohexane has shown better solubility. Acetic acid has not been very successful as a catalyst, but pivalic acid has shown good results. To remove the water from the reaction medium, a Dean-Stark apparatus was used, and anhydrous sodium sulfate was added occasionally.

Products were purified by column chromatography using a solvent gradient of $CHCl_3$:MeOH or hexane:AcOEt, with yields of 18-64%. All compounds were fully characterized by FTIR, MS and 1D and 2D NMR. For example, the ^{13}C NMR spectra showed the absence of both signals for carbonyl groups and the presence of an alkyl chain and aminoquinolinyl aromatic ring, proving the formation of the desired products.



Reagents and conditions: (i) pivalic acid, THF:C_6H_{12}, 80-100°C, 18-64%.

Scheme 1: Synthesis of the new pyrrole-atorvastatin analogues **4a-d**.

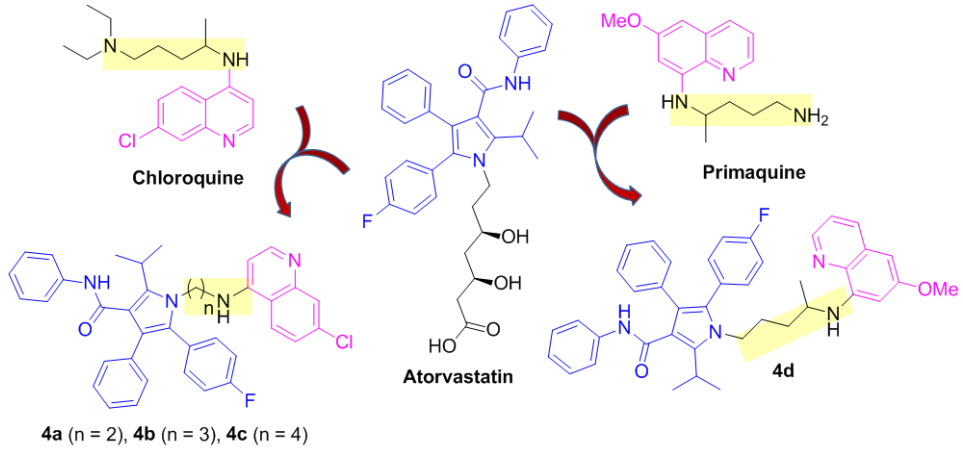
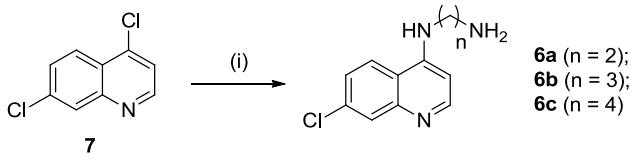


Figure 2: New pentasubstituted pyrrole hybrid atorvastatin-quinoline derivatives.

The diamino-7-chloroquinolinyl derivatives **6a-c** were synthesized by reflux for 4 hours of **7** and the corresponding diamine-alkanes of interest,[37,38] with yields of 91-96% (Scheme 2). Primaquine was obtained by the simple neutralization of primaquine diphosphate with 10% aqueous NaOH solution at room temperature.[39]



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Reagents and conditions: (i) appropriate diamine, reflux, 4 h, 91-96%.

Scheme 2: Obtaining of intermediary aminoquinolines**6a-c**.

P. falciparum parasites were cultured in human red blood cells under conditions established by Trager & Jensen (1976),[40] with minor modifications.[41] Briefly, parasites were cultured in Petri dishes (Corning, Santa Clara, CA, USA) with 5% hematocrit in RPMI culture medium supplemented with 1% (v / v) albumax II (Gibco, USA). Plates were maintained at 37°C, using the candle jar method. Daily exchanges of the culture medium were carried out and parasitaemia monitored in Giemsa-stained smears. The parasites were synchronized by sorbitol solution as described by Lambros & Vandenberg (1979)[42] to get predominantly ring forms, diluted and incubated in 96 well plates containing the test and control compounds, or culture medium with 0.5% DMSO, used as a positive control of parasite growth.

The SYBR test was used as previously described by Smilkstein *et. al*. (2004)[43] with some modifications, as follows. Briefly, the test compounds, in serial dilutions, were incubated with the parasite suspensions (0.5% parasitaemia and 2% hematocrit) in 96-wells plates ("U" bottom). After 48 h at 37°C, the culture supernatant was removed replaced by 100µL of lysis buffer solution [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)] followed by addition of 0.2 µL/mL Sybr Safe (Sigma-Aldrich, Carlsbad, CA, USA). The plate content was transferred to a flat bottom plate, then incubated in the dark for 30 minutes. The reading was made in a fluorometer (Synergy H4 Hibrid Reader, BioteK) with excitation 485 nm and emission of 535 mm.

For the test of cytotoxicity, a monkey kidney cell line (BGM), originally received from the Federal University of Minas Gerais was used and the test performed as described.[44] Cells were cultured in 75 cm^2 plates with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and gentamicine 40 mg/L, at 5% CO_2 atmosphere and 37°C. When the cell monolayer was confluent it was trypsinized, washed with culture medium, distributed in a flat-bottomed 96-well plate (1×10^5 cells/mL) and incubated for 18 h at 37°C to ensure cell adherence. The cells were properly diluted and incubated with 20 µL of the compounds at different concentrations (1,000-1 µg/mL) for 24 h in a 5% CO_2 and air atmosphere at 37°C. The neutral red assay[45] was used to evaluate cell viability by the accumulation of dye in the viable cell lysosome. 200 µL of neutral red solution (4 mg/mL), were added and the plates and incubated for 3 h. The supernatant was carefully removed, followed by the addition of 200 µL of formaldehyde (0.5% v/v) and $CaCl_2$ (1%) solution. After 5 min, the supernatant was removed, then 100 µL of an alcohol- acetic acid (50-1%) solution were added to extract the dye. The absorbance was read at 540 nm on an ELISA reader (SpectraMax340PC[384], Molecular Devices). Cell viability was expressed as the percentage of control absorbance obtained in untreated cells and the minimum lethal dose for 50% of the cells (MLD_{50}) was determined.

The results of the antiplasmodial activity and cytotoxicity assays of four new compounds (**4a-d**) are summarized in Table 1. All compounds synthesized showed activity against a W2-chloroquine resistant *P. falciparum* clone, with IC_{50} values ranging from 0.40 to 1.41 µM in the SYBR test, in a similar range to chloroquine (IC_{50} = 0.59 µM); in addition, all were better than primaquine (IC_{50} = 1.89 µM) and than atorvastatin (IC_{50} = 10.3 µM). Compounds **4a**, **4b** and **4c** were the most active, with IC_{50} values of 0.99, 0.65 and 0.40 µM, respectively. All compounds were also evaluated regarding cytotoxicity against a BGM cell line. Compounds **4a** and **4d** presented the lowest toxicity measured at doses up to 1,000 µg/mL with SI > 1,677 and 1,107, respectively.

Table 1. Evaluation *in vitro* of antiplasmodial activity against *Plasmodium falciparum* W2 clone (chloroquine resistant), cytotoxicity against a monkey kidney cell line (BGM), and drug selectivity index (SI) of compounds **4a–d**, **8**, chloroquine and primaquine.

Compounds	IC_{50} (µM)	MDL_{50} (µM)	SI (MDL_{50}/IC_{50})
4a	0.99 ± 0.3	> 1,661 ± 0.0	> 1,677
4b	0.65 ± 0.5	136.4 ± 14	210
4c	0.40 ± 0.02	187.3 ± 13	468
4d	1.41 ± 0.14	> 1,562 ± 0.0	> 1,107
8	6.39 ± 0.98	> 2,202	> 345
Chloroquine	0.59 ± 0.03	1,219	2,066
Primaquine	1.89 ± 0.2	451.7	239
Atorvastatin	10.3 ± 1.2	77	7.5

IC_{50}: inhibitory concentration for 50% of parasite growth, evaluated in two to four different experiments for each test; MDL_{50}: minimal dose lethal concentration for 50% of BGM cells; SI: selectivity index. The MDL of atorvastatin is based in one experiment.

The literature indicates that a side chain length of 3 or 4 carbons generally has better activity against *P. falciparum*, but the activity also depends on the structure of the terminal amine.[32,46] We have recently evaluated quinoline–sulfonamide hybrids, which have a 7-chloroquinoline moiety connected by a linker group to arylsulfonamide moieties[32]. The most active hybrids assayed against *P. falciparum* had four methylene groups as linkers. Herein, the activity of the compounds increased with the size of the carbon chain. Compound **4c** (n=4) displayed better activity even though the terminal amine is a tertiary amine with the electron lone pair impaired by the aromaticity of the pyrrole ring. Despite being the least active of the series, primaquine derivative **4d** (IC_{50}=1.41) was significantly less toxic and more active than primaquine, encouraging us to proceed in new searches for a substitute for this toxic drug.

Additionally, to evaluate the importance of the pyrrolic moiety in the hybrid compounds, compound **8** was obtained[47] (Fig. 3) using the same method as for the series **4a-d**. Compound **8** is a pentasubstitued pyrrole without the aminoquinolinyl moiety. No toxicity was found *in vitro* with doses up to 1,000 µg/mL (> 2,202 µM), and its activity (IC_{50} = 6.39 µM) is higher than that found for AVA (IC_{50} = 10.3 µM). This result suggests that the pentasubstituted pyrrole might be the pharmacophoric group of AVA. Comparing these new results for **4a**, **4b**, **4c** and **4d** with

the results found for AVA our compounds were 10, 16, 26 and 7-fold more active than AVA, respectively, showing the significance of the aminoquinolinyl attached to AVA's pyrrolic moiety connected by a linker with four methylenes.

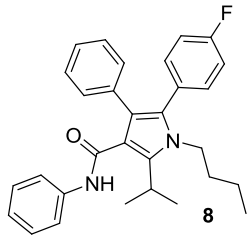

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Figure 3: Structure of compound **8**, a pentasubstituted pyrrole synthesized with no aminoquinoline moiety.

In summary, four new pyrrolic hybrids of AVA with aminoquinolines were synthesized and assayed against *P. falciparum*. None of these compounds was significantly toxic to BGM cells, although one of them (**4b**) was more toxic than the others and thus had the lowest selectivity index. All compounds showed anti-*P. falciparum* activity ranging from 0.40 to 1.41 µM in the SYBR test; they were almost 5-fold more active than primaquine. Compounds **4c** and **4d** (IC_{50} = 0.40 and 1.41 µM, respectively), with low IC_{50} values, were better than chloroquine and primaquine, respectively, with good SI values. Compound **4d** was shown to be safer (SI > 1,107) than primaquine (SI was 239).

New compounds containing pentasubstituted pyrrole-quinolines will be synthesized with the expectation of enhanced potency and solubility, and will also be assayed for cerebral antimalarial activity to clarify the importance of AVA in this scaffold.

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36. General procedure for the preparation of new pyrrole-atorvastatin analogues (**4a-d** and **8**): Diaminoquinolines (**6a-d**) (1.8 to 2.7 mmol) and 1,4-diketone 2-(2-(4-fluorophenyl)-2-oxo-1-phenylethyl)-4-methyl-3-oxo-N-phenylpentanamide (**5**) (1.5 mmol) were solubilized in a mixture 1:1 of cyclohexane and dry anhydrous THF. Pivalic acid (1.5 to 4.5 mmol) was added to the reaction medium, and the mixture was heated to 80°C for 24 to 48 hours. The mixture was then purified by column chromatography with a gradient of $CHCl_3$:MeOH or hexane:AcOEt.
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38. General procedure for the preparation of *N*-(7-chloro-4-quinolyl)-1,n-diaminoalkanes (**6a-c**): A mixture of 4,7-dichloroquinoline (5 mmol) (**7**) and 3 mL of diaminoalkane was heated to reflux for 4 h. The mixture was then poured into crushed ice, and the resulting pale-yellow precipitate was filtered under vacuum and used without further purification.
39. Procedure for the preparation of free base primaquine (**6d**): Primaquine diphosphate (4 mmol) was dissolved in the minimum amount of distilled water with constant magnetic stirring, then it was covered with aluminum foil. An aqueous solution of 10% sodium hydroxide was added slowly until the reaction medium reached pH = 10. The reaction was left under stirring and shielded from light at room temperature for 2 hours. The mixture was extracted with dichloromethane and dried over anhydrous sodium sulfate to give a brown oil.
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