Design, Synthesis and Evaluation of New Fluoroamodiaquine Analogues

Ana Carolina Corrêa de Sousa,¹ Gil Mendes Viana,¹ Nuria Cirauqui Díaz,² Marianne Grilo Rezende,³ Filipe Fernandes de Oliveira,⁴ Raquel Pinto Nunes,⁵ Monica Farah Pereira,⁶ André Luiz Lisboa Areas,⁶ Marianos Gustavo Zalis,⁶ Valber da Silva Frutuoso,⁷ Hugo Caire de Castro Faria,⁵ Thaisa Francielle Souza Domingos,⁵,⁶ Marcelo de Pádua,⁶ Lucio Mendes Cabral,*⁶ and Carlos Rangel Rodrigues⁶

¹ Laboratório de Tecnologia Industrial Farmacêutica (LabTIF), Faculdade de Farmácia, Universidade Federal do Rio de Janeiro; Rio de Janeiro, RJ 21941–902, Brasil: ² Laboratório de Modelagem Molecular e QSAR (ModMoQSAR), Faculdade de Farmácia, Universidade Federal do Rio de Janeiro; Rio de Janeiro, RJ 21941–902, Brasil: ³ Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz; Rio de Janeiro, RJ 21040–360, Brasil: ⁴ Laboratório de Infectologia e Parasitologia Molecular, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro; Rio de Janeiro, RJ 21941–913, Brasil: and ⁵ Laboratório de Microbiologia e Avaliação Genotóxica (LAMIAG), Faculdade de Farmácia, Universidade Federal do Rio de Janeiro; Rio de Janeiro, RJ 21941–902, Brasil. Received December 10, 2015; accepted February 19, 2016

Malaria is one of the most important tropical diseases; the use of amodiaquine as a current chemotherapy in the treatment of malaria has shown some problems such as hepatotoxicity and agranulocytosis. In this work we present the rational design, synthesis, and biological evaluation (antimalarial activity, cytotoxicity and genotoxicity) of four new fluoroamodiaquine analogues. The results showed significant correlation between MolDock score and IC₅₀ values. The molecules 7b and c were the most active of the planned compounds, with lower IC₅₀ against Plasmodium falciparum W2 strain (0.9 and 0.8 μM, respectively) and an excellent cytotoxicity profile. The present study revealed no mutagenicity or genotoxicity for the analogues. Confirming our docking results, the molecular dynamics showed that compound 7b remains stably bound to the heme group by means of π– stacking interactions between quinoline and the porphyrin ring. Based on these findings, this study may prove to be an efficient approach for the rational design of hemozoin inhibiting compounds to treat malaria.

Key words docking; molecular dynamics; molecular modeling; antimalarial

Malaria is one of the most important tropical diseases, affecting 97 countries. It is an infectious disease caused by five species of Plasmodium genus protozoa, from which the P. falciparum is the most lethal in humans. There were approximately 198 million cases of malaria reported in 2013, and an estimated number of 584 000 deaths, mainly in African sub-Saharan countries.¹

The chemotherapy combination of artesunate and amodiaquine (ASAQ) is currently the treatment recommended by WHO. However, recent reports show that P. falciparum has become resistant to these chemotherapeutic agents. In addition, the use of amodiaquine (AQ) (Fig. 1) has shown some problems such as hepatotoxicity and agranulocytosis.²–⁴ This is a result of the biotransformation that occurs in the liver by CYP450 enzymes, which generates a reative quinoneimine metabolite, the amodiaquine quinone imine (AQIQ)⁵ (Fig. 1). This metabolite binds irreversibly to cellular macromolecules, leading to cell death by oxidation and, probably, to direct toxicity as well as immune-mediated hypersensitivity reactions.⁶,⁷ The mechanism of action of AQ and other 4-aminoquinolines is based on the inhibition of the parasite’s mechanism of detoxification of heme, namely, the inclusion of free heme into hemozoin. By doing so, AQ increases the concentration of free heme inside the host cell acidic digestive vacuole, killing the parasite by oxidative stress.⁸–¹¹ The design of new amodiaquine derivatives with reduced toxicity and increased activity is a current matter of study.¹²–¹⁵ It’s already well established in the literature that the fluorine insertion in this series is favorable to reduce the hepatotoxicity, by forming new compounds that are more resistant to oxidation and hence less likely to form toxic quinone imine metabolites in vivo.²,³,¹⁶,¹⁷,¹⁸

O’Neill et al. demonstrated that the 4’-hydroxyl group could be replaced with a 4’-fluorine atom to produce an amodiaquine analogue, fluoroamodiaquine (FAQ), which retained the antimalarial activity while decreasing the formation of toxic quinone imine metabolites in vivo, thereby decreasing the compounds hepatotoxicity⁹ (Fig. 1). Moreover, the introduction of a piperezine moiety showed good activity against chloroquine resistant strains of P. falciparum.¹⁹,²⁰ In a previous work by our group, a molecular modeling study was applied to a data set of AQ derivatives, suggesting the structural features that are important for the interaction of compounds with heme.²¹ Taking together the information gained in this study, and that available in the literature, we present here the rational design (Fig. 1), synthesis (Chart 1), biological evaluation (antimalarial activity, cytotoxicity and genotoxicity) of four new FAQ analogues.

Results and Discussion

Molecular Docking The study of the binding between our compounds and the heme group was performed following a procedure published before.²¹ For the design of compounds using the Spartan’10 program, all calculations were performed on a Core 2 Quad personal computer, running Windows XP operational system.²² The predominant ionization state of
each compound in the pH range from 5.0 to 5.6 (i.e., in the acidic digestive vacuole of the parasite) was predicted using the Chemicalize server (http://www.chemicalize.org/). After building the three-dimensional (3D) structures, the free heme (Fe protoporphyrin IX) geometry was obtained from the met-hemoglobin crystal structure available on the Protein Data Bank (PDB ID: 3P5Q). The docking study was performed using the Molegro Virtual Docker (MVD) (version 2011.5.0).
program (Molegro ApS). The atom types and the bond orders were corrected using the MVD automatic preparation function. The hydrogen atoms were added and the MVD default atomic charges were assigned. To increase the docking precision the parameter of population size was changed for 100, the default parameters of maximum interactions, scaling factor, and crossover rate were kept. For each ligand, 100 independent docking runs were performed with the MolDock optimizer algorithm. The MolDock score function was used to pre-compute score grids for rapid energy evaluation. The solutions (poses) obtained for each independent run were reclassified using the Molegro re-rank scoring function, and the best scoring pose of each run was taken. Afterwards, a filter was applied to these 10000 solutions, in which those not consistent with the postulated mechanism of action were excluded.

Table 1 shows the MolDock and re-rank score energies for the complexes obtained with all ligands. As expected, the predicted binding affinity of the compounds was similar to that of amodiaquine. These data suggest that the new analogues could form stable non-covalent complexes with the heme group. Experimental and theoretical data indicates that the 4-aminoquinolines and the heme group interact mainly by π–stacking interactions between the quinolone and the porphyrin ring, and by a charge-assisted hydrogen bond interaction between a donor group in the 4-aminoquinolines and the heme carboxylate group. Moreover, Menezes et al. carried out a docking study between a series of pyrazolo-pyridine isosteres of mefloquine (4-methanol-quinolines) and the heme group, and the authors observed that the distance between the quinoline ligand moiety and the heme porphyrin rings was shorter for the most active compounds, correlating the π–stacking interaction with the inhibitory profile. They suggested an optimal distance value of approximately 3.0–4.0 Å.

According to our results, all our compounds perform π–stacking interactions with heme, showing a distance of 3.64 Å (7a), 3.25 Å (7b), 3.41 Å (7c) and 3.40 Å (7d) (Fig. 2). Concerning the charge-assisted hydrogen bond, compounds 7a, 7b and c present the protonated nitrogen of the piperazine moiety near the carboxilate heme group. In the 7b–heme complex, the charge-assisted hydrogen bond is observed with the ligand protonated nitrogen of piperazine moiety (3.36 Å) (Fig. 2). We can observe that in all the complexes the fluoro substitution prevent the hydrogen bond with the heme carboxylate group. Moreover, other electrostatic interactions were seen between some nitrogen atoms of the porphyrin ring and some NH group of the ligands.

**Chemistry** The new target compounds 7a–d were synthesized following the methodologies described by Guglielmo et al. and Gemma et al., as shown in Chart 1. The synthesis started with the commercial available 2-fluoro-5-nitrobenzyl alcohol 1, which was reduced with iron powder and hydrochloric acid to give 5-amino-2-fluorobenzyl alcohol 2 in excellent yield (90%). Compound 4, prepared from the reaction of 2 with 4,7-dichloroquinoline 3, could be converted to 5 with concentrated hydrochloric acid under reflux. Then, the novel intermediate 5 was treated with an excess of appropriate piperazines 6a–d at room temperature in acetonitrile, to afford the new desired compounds in moderate to excellent yields (54–89%).

**Biological Evaluation**

**Antiplasmodial Activity and Toxicity assay** The in vitro antiplasmodial activity of the fluoroadamiaquine analogues was evaluated against the the *Plasmodium falciparum* W2 strain (resistant to chloroquine). The potency
of the compounds, as indicated by their IC<sub>50</sub> values, is summarized in Table 2, and compared to amodiaquine (AQ). In parallel, the cytotoxicity of the compounds was evaluated against Vero cells (CC<sub>50</sub> values) (Table 2).

All tested compounds exhibited modest to good activities on the CQ-sensitive W2, with IC<sub>50</sub> ranging from 5.3 to 0.8 μM. The molecules 7b and c were the most active of the planned compounds, with the lower IC<sub>50</sub> (0.9 and 0.8 μM, respectively). Moreover, those compounds showed selectivity to the parasite (selectivity index (SI) >80). Compound 7d was the most cytotoxic and the less active on P. falciparum strains. The values allowed us to calculate the SI corresponding to the ratio of toxic and the less active on 7d.

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It’s important to notice that these compounds were planned to show a good antimalarial profile but with lower or none hepatotoxicity that are observed in amodiaquine used nowadays to malaria treatment. Previous studies have shown that introduction of fluorne into the aromatic nucleus enhanced stability of the carbon-fluorine bond, reduce the oxidative bioactivation by increasing the stability of the aryl ring to oxidation in vivo and thereby reduces hepatotoxicity. Once was observed that in the drug–heme complexes the fluore substitution prevented the possible hydrogen bond with the heme carboxylate group, we can see the impact on the activity when compared to AQ which in turn presents the best activity. So, a molecular modification can be done to provide the hydrogen donor to favor the charge-assisted hydrogen bond with the heme carboxylate in further studies, nevertheless, observing the toxicity.

Our docking results correspond and explain the biological activity observed. The lower is the docking energy, the better the antiplasmodial activity observed. The 7b heme-complex (IC<sub>50</sub> 0.9 μM) showed the smaller docking energy (−124.50), re-rank score (−63.48), π–stacking interaction distance (3.25 Å) and exhibited the charge-assisted hydrogen bond between the protonated nitrogen of piperezine moiety and the heme carboxylate. Together with its good citotoxicity profile, it makes it a good candidate for further pharmacological studies and it was chosen to make the molecular dynamics studies in this work.

In Vitro Genotoxicity and Mutagenicity Assays Mutagenicity and carcinogenicity are among the toxicological effects that cause the highest concern for human health; and thus they are object of research in early stages of drug development. Studies of mutagenic and genotoxic effects of three compounds, namely chloroquine, primaquine and amodiaquine, show a weak but significant mutagenic effect in Salmonella strains at high concentrations. These tests are important since they provide information for predicting potential heritable germ cell damage as well as potential carcinogenicity.

The present study revealed no mutagenicity and genotoxicity for amodiaquine and the four fluoroamodiaquine analogues at the tested concentrations in assays without metabolic activation (spot test) (Table 3). Mutagenicity assay (Ames test) in the presence of S9 mix was performed with the analogue 7b, which presented the best biological activity. The results indicated that there was no statistically significant increase in the number of revertants for the S. typhimurium TA98 or TA100 strain at concentrations of 50 to 1000 μM/well (Table 4).

Molecular Dynamics In order to confirm our docking results, testing the stability of the 7b heme-complex, energy minimization and molecular dynamics (MD) simulations were performed with the GROMACS package version 4.5.4, using the gromos53a6 force field. The ligand topology was created with the PRODRG server, but it was manually corrected, and AM1 charges calculated by the UCSF Chimera package were assigned. MD simulations were carried out on a Linux in this work.

### Table 2. Antiparasitic Activity (IC<sub>50</sub>, µM) Tested against the Plasmodium falciparum W2 Strain (Resistant to Chloroquine), Cytotoxicity (Vero Cells, CC<sub>50</sub>, µM), and Selectivity Index (SI) of Amodiaquine (AQ) and the 7a–d Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>SI</th>
</tr>
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<tbody>
<tr>
<td>AQ</td>
<td>0.05</td>
<td>48.6 (±2.7)</td>
<td>972</td>
</tr>
<tr>
<td>7a</td>
<td>4.9</td>
<td>&gt;80</td>
<td>16.33</td>
</tr>
<tr>
<td>7b</td>
<td>0.9</td>
<td>&gt;80</td>
<td>88.89</td>
</tr>
<tr>
<td>7c</td>
<td>0.8</td>
<td>&gt;80</td>
<td>100</td>
</tr>
<tr>
<td>7d</td>
<td>5.3</td>
<td>68.8 (±4.5)</td>
<td>12.98</td>
</tr>
</tbody>
</table>

### Table 3. Mutagenic and Genotoxic Activity of Fluoroamodiaquine Analogue without Metabolic Activation (Spot Test) Evaluated by Ames Test and SOS Chromotest

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Ames test</th>
<th>SOS chromotest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. typhimurium</td>
<td>E. coli</td>
</tr>
<tr>
<td>TA97</td>
<td>TA98</td>
<td>TA100</td>
</tr>
<tr>
<td>7a</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7b</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7c</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7d</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>AQ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4-NQO</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DMSO</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

The fluoroamodiaquine analogues were dissolved in dimethyl sulfoxide (DMSO) to perform the assays. As positive control 4-NQO was used. Results of three different concentrations (10, 100, 500 μM) were presented. The S9 mix was added. Results are expressed as revertants for the tester strains TA98 or TA100.

### Table 4. Mutagenic Effect of Fluoroamodiaquine Analogue 7b with Metabolic Activation (S9 Mix) Observed in the Ames Test

<table>
<thead>
<tr>
<th>Dose per plate</th>
<th>S9 mix (4%)</th>
<th>Number of his&lt;sup&gt;+&lt;/sup&gt; revertent colonies/plate (mean±S.D.&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA98</td>
<td>TA100</td>
</tr>
<tr>
<td>SPTN 10 µg</td>
<td>−</td>
<td>18±2.79</td>
</tr>
<tr>
<td>2-AF 10 µg</td>
<td>−</td>
<td>24±4.96</td>
</tr>
<tr>
<td>2-AF 10 µg</td>
<td>+</td>
<td>2780±240.05</td>
</tr>
<tr>
<td>DMSO 1000 µM</td>
<td>+</td>
<td>22±2.82</td>
</tr>
<tr>
<td>7b 50 µM</td>
<td>+</td>
<td>20±2.21</td>
</tr>
<tr>
<td>7b 100 µM</td>
<td>+</td>
<td>21±2.87</td>
</tr>
<tr>
<td>7b 500 µM</td>
<td>+</td>
<td>22±1.50</td>
</tr>
<tr>
<td>7b 1000 µM</td>
<td>+</td>
<td>22±3.59</td>
</tr>
<tr>
<td>AQ 1000 µM</td>
<td>+</td>
<td>22±1.82</td>
</tr>
</tbody>
</table>

The fluoroamodiaquine analogue 7b was dissolved in dimethyl sulfoxide (DMSO) to perform the assay. As positive control, 2-AF was used. Results are expressed as revertants for the tester strains TA98 or TA100.
cluster with 20 cores (2.4 GHz, 32 GB). The SPC/E water model was used to solvate the complex in a cubic water box. All simulations were performed in periodic boundary conditions. The electrostatic interactions were treated by a combination of the Particle Mesh Ewald (PME) method and a switch function. Electrostatic and van der Waals interactions were considered to a cut-off of 1 nm, and the simulation time-step was set to 2 fs. The initial energy minimization (EM) was performed with the steepest-descent algorithm. Simulations of solvent molecules and counterions were performed at 310 K and 1 atm for 100 ps, with the compounds atoms restrained with harmonic potentials. Afterwards, the system was heated from 50 to 310 K using six blocks of calculation in which the temperature was gradually increased. Each block lasted 20 ps, totaling 120 ps of the heating process. The calibrated system was simulated until a total time of 200 ns.

Confirming our docking results, compound 7b remains stably bound to the heme group by means of \( \pi \)-stacking interactions between the quinoline and the porphyrin ring (Fig. 3). However, the piperazine tail remains highly flexible during the whole trajectory, and no interaction is observed between this tail and the heme group (Fig. 3b). This high flexibility increases the entropy of the system and, by doing so, the total energy of binding. The charge assisted hydrogen bond with the heme carboxylate group is performed by the ligand NH group in the quinolone (Fig. 3a). This same binding mode had been already found for the most active compound of the previous series studied by our group. Therefore, all our MD studies suggest that the substituent in the piperazine ring of the ligand do not modify the activity by a direct interaction with heme, but by means of other mechanisms, as could be the modification of the electronic distribution of the charge that will influence the distance of the \( \pi \)-stacking interactions.

Conclusion

The present study allowed the use of computational resources such molecular docking to a rational design before experimental data using the MVD program. Doing so, the novel target compounds 7a–d was synthesized with moderate to excellent yields (54–89\%). The molecules 7b and e were the most active of the planned compounds, with the lower IC\(_{50}\) (0.9 and 0.8 \( \mu \)M, respectively) and an excellent citotoxicity profile. The results showed significant correlation between MolDock score and IC\(_{50}\) values. All the results indicate that the molecules 7b and e are good candidates for further pharmacological studies. Moreover this study may prove to be an efficient approach for the rational design of hemozoin inhibiting compounds to malaria treatment.

Experimental

Chemistry All chemicals were obtained from commercial suppliers and were used without further purification. \(^1\)H- and \(^13\)C-NMR spectra were recorded on an Avance 200 MHz spectrometer (Bruker) using CDCl\(_3\), methanol-\( d_4 \) and DMSO-\( d_6 \) as the solvent. Standard Bruker software was used throughout. Chemical shifts were given in ppm (\( \delta \) scale) and coupling constants (\( J \)) were given in hertz (Hz). The IR spectra were obtained on a IRPrestige-21 FTIR spectrometer (Shimadzu, Tokyo, Japan) using KBr pellets. High-resolution mass spectra (HR-MS) were obtained on a Bruker microTOF II mass spectrometer using electrospray ionization (ESI). Melting points were recorded on a Shimadzu DSC-60 thermal analyzer at a heating rate of 10°C/min, room temperature to 200°C, under a nitrogen flow rate of 50 mL/min and using an aluminum standard. Analytical TLC (silica gel, aluminum sheets 60 F254, Merck) was performed using ethyl acetate–hexane (3:1 v/v) as the eluent.

5-Amino-2-fluorobenzyl Alcohol (2)\(^{17}\) A mixture of 2-fluoro-5-nitrobenzyl alcohol 1 (2.00 g, 11.9 mmol), iron powder (3.72 g, 66.8 mmol) and hydrochloric acid (265.84 mg, 7.28 mmol) was heated under reflux for 2 h. The hot mixture was filtered in Celite and allowed to reach room temperature. This aqeous phase was then basified with a saturated solution of sodium bicarbonate and extracted with CH\(_2\)Cl\(_2\). The organic layer was separated, dried (Na\(_2\)SO\(_4\)), filtered, and concentrated by rotary evaporation to obtain the crude product as brown solid (1.51 g, 10.7 mmol, 90% yield). IR (KBr): 3568, 3410, 2930, 1628, 1512, 1439, 1358, 1036, 874, 818, 737 cm\(^{-1}\); \(^1\)H-NMR (CDCl\(_3\)) \( \delta \): 7.26 (brs, 2H), 6.84 (t, \( J=9.2 \) Hz, 1H), 6.75–6.69 (m, 1H), 6.61–6.50 (m, 1H) 4.67 (s, 2H).

(5-(7-Chloroquinolin-4-ylamino)-2-fluorophenyl)methanol (4)\(^{17}\) To a solution of 5-amino-2-fluorobenzyl alcohol 2 (1.40 g, 9.9 mmol) in ethanol (70 mL) 4,7-dichloroquinoline 3 (1.96 g, 9.92 mmol) was added and the mixture was refluxed

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Fig. 3. Results of the MD Simulations of the 7b Heme-Complex

a) Final binding mode (conformation closer to the average of the production run); b) Main clusters found along the MD trajectory, representing the main complex conformations. Non-polar hydrogen atoms were omitted for better viewing. The figure was created with the PyMOL program, version 1.5.0.4 Schrödinger, LLC.
for 2.5 h. The cooled reaction was filtered on a Buchner funnel to give the desired product (1.26 g, 90%) as yellow solid. IR (KBr): 3319, 3198, 3061, 2810, 1611, 1539, 1447, 1362, 1215, 1111, 1051, 887, 827, 787, 706 cm⁻¹; ¹H-NMR (DMSO-d₆) δ: 11.28 (brs, 1H), 8.93 (d, 1H, J = 9.1 Hz), 8.52 (d, 1H, J = 7.0 Hz), 8.20 (d, 1H, J = 2.0 Hz), 7.83 (dd, 1H, J = 1.6 Hz, 9.0 Hz), 7.58–7.53 (m, 1H), 7.47–7.27 (m, 2H), 6.73 (d, 1H, J = 7.0 Hz), 4.60 (s, 2H).

7-Chloro-N-(3-(chloromethyl)-4 fluorophenyl)quinolin-4-amine (5) A mixture of benzyl alcohol (4 (1.20 g, 8.5 mmol) and 160 mL of concentrated hydrochloric acid was heated at reflux for 18 h and the reaction was monitored by TLC. The reaction mixture was evaporated under reduced pressure and the crude product was triturated with ethyl ether and filtered to give the product (0.98 g, 70 mmol, 82%). Yellow solid; mp 270°C (dec.); IR (KBr): 3441, 3444, 2999, 2903, 2745, 2573, 1609, 1585, 1543, 1499, 1450, 1096, 907, 816, 785 cm⁻¹; ¹H-NMR (CDCl₃) δ: 11.28 (brs, 1H), 8.89 (d, 1H, J = 9.0 Hz), 8.49 (d, 1H, J = 6.6 Hz), 8.17 (s, 1H), 7.83–7.30 (m, 4H), 6.68 (d, 1H, J = 6.6 Hz), 4.81 (s, 2H); ¹³C-NMR (DMSO-d₆) δ: 162.0, 157.0, 155.5, 144.0, 139.7, 138.9, 134.0, 133.9, 129.0, 127.9, 119.8, 118.1, 117.6, 116.5, 100.8, 40.2; HR-MS-ESI: m/z [M+H]+ Calcd for C₉H₇N₂Cl₂F: 321.0356. Found: 321.0361.

Preparation of 7a–d Derivatives (General Procedure) A solution of compound 5 (0.20 g, 0.62 mmol) in acetonitrile (10 mL) was prepared, then N-substituted piperazine 6 (1.25 mmol) and triethylamine (0.3 mL, 2.07 mmol) were added nitrile (10 mL) was prepared, then N-substituted piperazine (1.25 mmol) and triethylamine (0.3 mL, 2.07 mmol) were added.

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Pale yellow solid; mp 270°C (dec.); IR (KBr): 3441, 3444, 2999, 2903, 2745, 2573, 1609, 1585, 1543, 1499, 1450, 1096, 907, 816, 785 cm⁻¹; ¹H-NMR (CDCl₃) δ: 11.28 (brs, 1H), 8.89 (d, 1H, J = 9.0 Hz), 8.49 (d, 1H, J = 6.6 Hz), 8.17 (s, 1H), 7.83–7.30 (m, 4H), 6.68 (d, 1H, J = 6.6 Hz), 4.81 (s, 2H); ¹³C-NMR (DMSO-d₆) δ: 162.0, 157.0, 155.5, 144.0, 139.7, 138.9, 134.0, 133.9, 129.0, 127.9, 119.8, 118.1, 117.6, 116.5, 100.8, 40.2; HR-MS-ESI: m/z [M+H]+ Calcd for C₉H₇N₂Cl₂F: 321.0356. Found: 321.0361.

Preparation of 7a–d Derivatives (General Procedure) A solution of compound 5 (0.20 g, 0.62 mmol) in acetonitrile (10 mL) was prepared, then N-substituted piperazine 6 (1.25 mmol) and triethylamine (0.3 mL, 2.07 mmol) were added successively at 0°C. The reaction was stirred at 0°C for 1 h and then, at room temperature, for 18 h. To the reaction mixture 4 mL of water was added and the suspension was allowed to stir at room temperature for 20 min until a yellow precipitate is formed. The solid was filtered on a Buchner funnel to give the crude product, which was purified by preparative chromatography.

7-Chloro-N-(4-fluoro-3-((4-(pyridin-2-yl)piperazin-1-yl)-methyl)phenyl)quinolin-4-amine (7a) Pale yellow solid; mp 85–87°C; IR (KBr): 3428, 2832, 2380, 1599, 1572, 1501, 1437, 1371, 1312, 1238, 1206, 1153, 1155, 980, 941, 854, 822, 772, 638, 554 cm⁻¹; ¹H-NMR (CDCl₃) δ: 8.48 (d, 1H, J = 5.3 Hz), 8.21–8.13 (m, 1H), 7.99–7.94 (m, 2H), 7.53–7.34 (m, 3H), 7.31–7.03 (m, 3H), 6.77 (d, 1H, J = 5.4 Hz), 6.64–6.57 (m, 2H), 3.63 (s, 2H), 3.55–3.42 (m, 4H), 2.60–2.47 (m, 4H); ¹³C-NMR (CDCl₃) δ: 161.4; 159.7; 156.5; 151.6; 149.3; 149.0; 148.1; 137.7; 135.8; 135.5; 134.5; 128.5; 126.3; 122.0; 118.0; 116.9; 116.5; 113.6; 107.3; 102.0; 55.2; 52.9; 45.4; HR-MS-ESI: m/z [M+H]+ Calcd for C₂₆H₂₄N₆Cl₂F: 447.1746. Found: 447.1742.

Biological Evaluation Antiplasmodial Activity The antimalarial activity was tested in vitro using W2 (chloroquine resistant) strains. All the parasites were maintained in continuous culture of human erythrocytes (blood group A+) using RPMI medium supplemented with 10% of inactivated human plasma.35 For the assays, parasites were synchronized for the ring stage with sorbitol 5% and the parasitemia was diluted to 0.5 and 2% hematocrit. The samples (sulubilized in dimethyl sulfoxide (DMSO)) was diluted in complete medium in various concentrations and incubated for 48 h. Amodiaquine and was used as standard antimalarial. The cultures were assessed by fluorescent assay previously reported.36 Briefly, the cells were lysed with an erythrocyte lysis buffer containing Sybr green and left for 1 h at 37°C. Later, the plates were read in fluorescence counter with excitation of 485 nm and emission of 535 nm and cut off 530 nm.

Toxicity Assay The Vero cell line (line of African Green monkey kidney cells) were analyzed by cell viability assay based on 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which evaluate mitochondrial reduction activity, according to Mossman.37 In a 96 well plate, the cells were cultivated (10⁴ cells/well) with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (200 µL/well). The cells were cultivated for 24 h at 37°C and 5% CO₂ to cell growth and then, the culture medium was aspirated and replaced by culture medium treated with the compounds in serial concentrations, with DMSO at fixed concentration of 1%. The compounds were tested in serial concentrations (2, 10, 40, 80 µM) and the cells were subjected to 24 h of exposure under the above conditions. After exposure, the cells were treated with MTT aqueous solution (2, 5 mg/mL)–100 µL of Hanks’ balanced salt solution (HBSS) pH 7.4 and 25 µL of the MTT solution per well. The plate was incubated for 3 h at 37°C and 5% CO₂. After the incubation time, the MTT solu-
tion was removed and the cells were washed with phosphate buffer pH 7.4. The buffer was replaced by DMSO (100µL/ well) to promote cell membranes rupture, allowing the release of formazan crystals formed, producing a more or less intense violet color according to the degree of cell viability. The absorbance reading were performed in Microplate Reader model TP-Reader NM, at 570 nm with reference at 690 nm, after vigorous shaking for 30 s. The absorbance data were analyzed using the statistical package Excel (Microsoft Office Excel 2007. Ink), by drawing a linear trend line correlating cell viability and tested concentration of the compounds, with \( R^2 \) above 0.99.

**In Vitro Genotoxicity and Mutagenicity Assays**

Reverse Mutagenesis to Histidine Prototrophy (Ames Test)

This assay was performed as described by Maron and Ames,\(^{(30)}\) using the histidine Salmonella typhimurium auxotrophic mutant strains TA97, TA98, TA100 and the wild type strain TA102, with and without metabolism. Different doses of fluoroamodiaquine analogues were assayed. All of them were diluted in DMSO. For metabolic activation, 0.5 mL of 4% S9 mix was added per plate. The metabolic activation mixture (S9) was freshly prepared before each test. Each assay was conducted in triplicate and for classifying any analogue as a mutagen the number of revertant colonies induced by it should at least be double of the number that is seen in the solvent control. In experiments without S9 mix (spot test) the number were compared with the solvent control by Student’s t-test. Positive control 4-NQO.

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**SOS Chromotest—“Spot Test”**

The SOS chromotest (spot test) was performed according to Quillardet and Hofnung,\(^{(39)}\) using Escherichia coli strains PQ35 and PQ37. One hundred microliters of an overnight culture of the E. coli strains are diluted in 5 mL of LB medium and the culture is incubated at 37°C in a gyratory incubator up to a concentration of \(2 \times 10^{10}\) bacteria/mL. Fractions of 0.1 mL of the culture are then distributed into test tube with top agar, and the mixture is poured immediately on M63 medium plate. A sample of 10 µL of the analogues is spotted onto the center of the plate. After overnight incubation at 37°C, the presence of a blue ring around a zone of inhibition indicates genotoxic activity. Each assay was conducted in triplicate and the results obtained show a comparison between the analogues and the positive control 4-NQO.

**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.

**References**


