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Halogenation as a Strategy to Improve Antiplasmodial Activity: A Report of New 3-Alkylpyridine Marine Alkaloid Analogs

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Abstract

Introduction: Due to the emergence of resistance to antimalarial drugs as well as the lack of vaccination for malaria, there is an urgent demand for the development of new antimalarial alternatives. Recently, our research group developed a new set of 3-alkylpyridine marine alkaloid analogs, of which a compound known as compound 5 was found to be inactive against *Plasmodium falciparum*.

Methods: Herein, we report a successful halogenation strategy to improve the antiplasmodial activity of compound 5 through the replacement of a hydroxyl group by chlorine (compound 6) and fluorine (compound 7) atoms.

Results: Compounds 6 and 7 showed improved antiplasmodial activities (IC_{50} = 7.2 and 8.3 μM, respectively) 20 times higher than that of their precursor, compound 5 (IC_{50} = 210.7 μM). Ultraviolet-visible titration experiments demonstrated that halogenation of compound **5** did not alter its ability to bind its target, hematin.

Conclusion: Halogenation can enhance the antiplasmodial activity of a compound without altering its mechanism of action.

Keywords: *Plasmodium falciparum*, 3-Alkylpyridine Marine Alkaloid Analogs, Antiplasmodial Activity, Halogenation, Malaria

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Introduction

According to the World Health Organization (WHO), despite the notable decrease in malaria burden from 2010 to 2015 (from 239 to 214 million cases), a slight increase in the number of malaria cases over the last few years suggests that progress in malaria eradication has stalled. In 2017, an estimated 219 million cases occurred worldwide. In the same year, an estimated 435 thousand deaths globally were attributable to malaria, with children under 5 years of age accounting for 61% (266.000) of all deaths.[1] Meanwhile in Brazil, the number of malaria cases increased by 48% in 2017 compared to 2016.[2] Malaria in humans is caused by different *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. Recently, it was found that humans can also be infected with *Plasmodium knowlesi*, a species that infects primates.[3] Clinical manifestations of malaria in humans are associated with its intraerythrocytic cycle. For example, the high rate of sequestration of *P. falciparum*-infected erythrocytes is a feature of severe *P. falciparum* infection.[4]

There is a high need for the development of new antimalarial agents due to the emergence of parasite drug resistance, including resistance to artemisinin-based combination therapy (ACT) and vector insecticide resistance, as well as the lack of an effective vaccine.[5] ACT is the therapy recommended by the WHO for the treatment of uncomplicated *falciparum* malaria, and there is a broad consensus that its efficacy needs to be protected.[1]

Regarding this concern, an increasing amount of investment is being made by domestic and international sources since

2000.[1] As a result, the process of identifying new antimalarial candidates has evolved, leading to 13 new antimalarial drugs in drug development, nine of which were in Phase 2 in 2018.[6]

For the last 7 years, our research group has been studying the antiplasmodial activity of synthetic analogs of theonelladin C, a 3-alkylpyridine marine alkaloid (3-APA), some of which have exhibited promising antiplasmodial activity.[7] Moreover, by adopting *in silico* simulation and biophysical techniques, our research group proposed that the mechanism for the antimalarial action of these analogs is through interference with the process of hemozoin formation.[8]

Some 3-APA analogs with a short alkyl chain, however, were considered inactive against *P. falciparum in vitro* (in-house library of compounds). As an example, compound **5** exhibited a high IC_{50} value (210.7 µM) but was found to interact with hematin in a pattern similar to that of chloroquine (CQ) (Figures 1a and 1b). This paradoxical behavior of compound **5**, its ability to form a complex with hematin, and at the same time, its inactivity against *P. falciparum* suggests its inability to reach its target *in vitro*.

Starting from this point, in an attempt to improve the permeability of compound **5** and, consequently, its ability to reach the parasite's hematin, the hydroxyl group was replaced with chlorine and fluorine atoms (the detailed synthetic route is described in Supporting Information, item 1). This strategy, known as halogenation, may improve some of the compound's pharmacological properties such as membrane permeation, metabolic stability, and target affinity.[9]

Methods

Chemicals and Reagents

1,6-hexanediol;3,4-dihydro-2*H*-pyran(DHP);toluene;sodium hydrogen sulfate ($NaHSO_4$); hexane; sodium sulfate (Na_2SO_4); silica gel 60 (SiO_2); ethyl acetate (EtOAc); dichloromethane (CH_2Cl_2); triethylamine (Et_3N); methanesulfonyl chloride (MsCl); tetrabutylammonium bromide ($Bu_4N^+Br^-$); diethyl ether (Et_2O); sodium hydroxide (NaOH), methanol (MeOH); hydrochloric acid (HCl); *N*-chlorosuccinimide (NCS); triphenylphosphine (PPh_3); (diethylamino) sulfur trifluoride (DAST); sodium bicarbonate ($NaHCO_3$); RPMI 1640 medium; sorbitol; 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT); fetal bovine serum (FBS); penicillin; streptomycin; dimethylsulfoxide (DMSO); hematin chloride; HEPES; tetramethylsilane (TMS); potassium bromide (KBr); deuterochloroform ($CDCl_3$).

Chemistry

Reagents and solvents were purchased as reagent grade and used without further purification. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts are reported as δ (ppm) downfield from TMS, and the *J* values are reported in Hz. IR spectra were recorded using a Shimadzu IRAffinity-1 Fourier transform spectrometer (Shimadzu Corp., Kyoto, Japan). Low-resolution mass spectra (LRMS) were recorded using an ESI Bruker Daltonics amaZon SL Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Column chromatography was performed with SiO_2, 70–230 mesh (Merck, Darmstadt, Germany).

Procedure for the Synthesis of 6-(oxan-2-yloxy)hexan-1-ol (2)

A mixture of 1,6-hexanediol 1 (1.0 equiv.), DHP-toluene (5% v/v) (1,96 equiv.), and aqueous 5 M $NaHSO_4$ (0.1 mL) was prepared. This mixture was stirred for at 30 ˚C for 3 hours and then extracted with hexane (3 times). The combined organic phases were dried with Na_2SO_4, filtered, and then evaporated under reduced pressure. The residue obtained was chromatographed (SiO_2, hexane/EtOAc 80:20) to yield pure compound 2.

6-(oxan-2-yloxy)hexan-1-ol 2: Yield 79%, *Rf* = 0.29 (hexane/EtOAc 80:20), colorless oily product: IR (KBr): $\overline{\nu}$ = 3398, 2939, 2865, 1406, 1440, 1138, 1120, 857 cm^{-1}. ^1H NMR

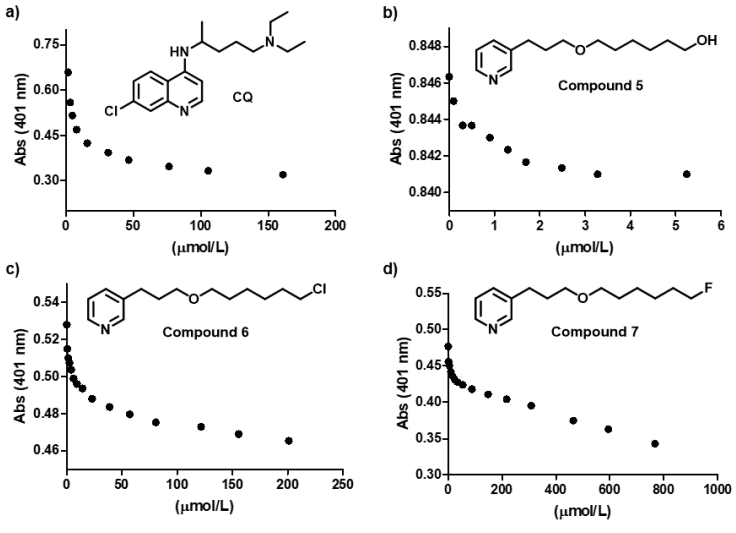

Figure 1. Titration of Hematin (5.0 µmol/L) With Increasing Concentrations of **a)** CQ (0-161.0 µmol/L) (inset shows a plot of absorbance at 401 nm versus the concentration of CQ); **b)** compound 5 (0-5.24 µmol/L) (inset shows a plot of absorbance at 401 nm versus the concentration of compound 5); **c)** compound 6 (0-201.0 µmol/L) (inset shows a plot of absorbance at 401 nm versus the concentration of compound 6); and **d)** compound 7 (0-767.0 µmol/L) (inset shows a plot of absorbance at 401 nm versus the concentration of compound 7).

($CDCl_3$, 400 MHz): δ 1.33-1.42 (m, 4H), 1.50-1.61 (m, 8H), 1.69-1.75 (m, 1H), 1.79-1.87 (m, 1H), 3.39 (dt, 1H, J = 9.6 Hz, J = 6.5 Hz), 3.48-3.53 (m, 1H), 3.64 (t, 2H, J = 6.6 Hz), 3.74 (dt, 1H, J = 9.6 Hz, J = 6.8 Hz), 3.84-3.90 (m, 1H), 4.57 (t, 1H, J = 2.6 Hz) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.69, 25.45, 25.52, 26.00, 29.65, 30.75, 32.66, 62.85, 67.49, 98.88 ppm. LRMS (ESI) *m/z* 225.0 $[M+Na]^+$.

Procedure for the Synthesis of 6-(oxan-2-yloxy)hexyl methanesulfonate (3)

A solution of monotetrahydropyranyl acetal 2 (1.0 equiv.) in CH_2Cl_2 (50 mL) was cooled to 0°C. Et_3N (4.0 equiv.), and MsCl (2.0 equiv.) was added. The reaction mixture was stirred for 24 hours at room temperature and then poured into crushed ice and extracted with CH_2Cl_2 (3 times). The organic layer was dried with Na_2SO_4, filtered, and evaporated under reduced pressure. The residue obtained was purified by column chromatography (SiO_2, hexane/EtOAc 1:1) to yield pure compound 3.

6-(oxan-2-yloxy)hexyl methanesulfonate 3: Yield 82%, *Rf* = 0.25 (hexane/EtOAc 80:20), yellow oily product: IR (KBr): $\overline{\nu}$ = 2929, 2865, 1420, 1354, 1174, 1124, 1122, 856 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.37-1.44 (m, 4H), 1.52-1.60 (m, 6H), 1.72-1.79 (m, 1H), 1.80-1.86 (m, 1H), 3.01 (s, 3H), 3.39 (dt, 1H, J = 9.6 Hz, J = 6.4 Hz), 3.48-3.53 (m, 1H), 3.74 (dt, 1H, J = 9.6 Hz, J = 6.7 Hz), 3.84-3.89 (m, 1H), 4.23 (t, 2H, J = 6.5 Hz), 4.57 (tz 1H, J = 2.6 Hz) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.71, 25.27, 25.44, 25.72, 29.05, 29.50, 30.74, 37.34, 62.44, 67.32, 70.01, 98.92 ppm. LRMS (ESI) *m/z* 303.0 $[M+Na]^+$.

Procedure for the Synthesis of 1-[3-(pyridin-3-yl)propoxy]-6-(oxan-2-yloxy)hexane (4)

A mixture of 3-(pyridin-3-yl)propan-1-ol (1.0 equiv.) and $Bu_4N^+Br^-$ (0.33 equiv.) was prepared in 20 mL of Et_2O with 5 mL of aqueous NaOH (50% v/v) and stirred for 15 minutes. After this period, the mesylated compound 3 (1.0 equiv.) was added. The mixture was stirred vigorously at room temperature for 72 hours and then extracted with Et_2O (3 times). The combined organic layers were dried with Na_2SO_4, filtered, and evaporated under reduced pressure. The residue obtained was chromatographed (SiO_2, hexane/EtOAc 1:1) to yield pure compound 4.

1-[3-(pyridin-3-yl)propoxy]-6--(oxan-2-yloxy)hexane 4: Yield 60%, *Rf* = 0.44 (hexane/EtOAc 1:1), yellow oily product: IR (KBr): $\overline{\nu}$ = 2929, 2865, 1456, 1421, 1124, 1120, 983, 857 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.30-1.43 (m, 8H), 1.50-1.63 (m, 4H), 1.66-1.75 (m, 1H), 1.79-1.92 (m, 1H), 2.70 (t, 2H, J = 7.4 Hz), 3.38-3.43 (m, 5H), 3.47-3.53 (m, 1H), 3.74 (dt, 1H, J = 9.6 Hz, J = 6.8 Hz), 3.84-3.90 (m, 1H), 4.58 (t, 1H, J = 2.6 Hz), 7.19-7.23 (m, 2H), 7.52 (d, 2H, J = 7.8 Hz), 8.43-8.46 (m, 2H) ppm. ^{13}C NMR ($CDCl_3$, 100MHz): δ 19.67, 25.45, 26.09, 29.48, 29.67, 30.74, 30.97, 62.33, 67.53, 69.40, 70.94, 98.83, 123.22, 135.84, 137.19, 147.27, 149.97 ppm. HRMS $[M+Na]^+$ = 344.2193 (theoretical 344.2202).

Procedure for the Synthesis of 6-[3-(pyridin-3-yl)propoxy] hexan-1-ol (5)

To a solution of compound 4 (1.0 equiv.) in MeOH (50 mL) was added 3.36 mL of 1 M HCl (2.0 equiv.). After the addition, the reaction was stirred for 12 hours at room temperature, and the solution was concentrated under reduced pressure. Equal volumes of EtOAc and distilled water were added, and the pH was brought to 10 with the addition of 2 M NaOH. The solution was then extracted with EtOAc (3 times). The combined organic layers were dried with Na_2SO_4, filtered, and evaporated under reduced pressure. The residue obtained was chromatographed (SiO_2, EtOAc 1:1) to yield pure compound 5.

6-[3-(pyridin-3-yl)propoxy]hexan-1-ol **5**: Yield 70%, *Rf* = 0.33 (EtOAc), yellow oily product: IR (KBr): $\overline{\nu}$ 3348, 2927, 2854, 1457, 1423, 1357, 1130, 1114, 985, 856 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.23-1.42 (m, 4H), 1.56-1.63 (m. 4H), 1.89 (m, 2H), 2.71 (t, 2H, J = 7.4 Hz), 3.38-3.42 (m, 4H), 3.65 (t, 2H, J = 6.5 Hz), 7.20-7.24 (m, 1H), 7.52 (d, 1H, J = 7.8 Hz), 8.42-8.45 (m, 2H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 25.53, 25.95, 29.40, 29.61, 30.85, 32.70, 62.66, 69.28, 70.80, 123.30, 135.97, 137.22, 147.12, 149.86 ppm. HRMS $[M+H]^+$ = 238.1801 (theoretical 238.1807).

Procedure for the Synthesis of 3-(3-((6-chlorohexyl)oxy) propyl)pyridine (6)

To a solution of NCS (1.1 equiv.) dissolved in CH_2Cl_2, cooled at 0°C, and under inert atmosphere, a solution of PPh_3 (1.1 equiv.) dissolved in CH_2Cl_2 was added. This solution was stirred for 10 min followed by the addition of a solution of compound 5 (1.0 equiv.). The solution was allowed to warm to room temperature and stirred for 18 hours. After this period, the solvent was evaporated under reduced pressure. Subsequently, a small volume of Et_2O was added; the recipient was placed in the refrigerator for precipitation of PPh_3. The mixture was filtered and evaporated. The residue was purified by column chromatography (SiO_2, EtOAc/MeOH 95:5) to yield pure compound 6.

3-(3-((6-chlorohexyl)oxy)propyl)pyridine **6**: Yield 11%, *Rf* = 0.75 (EtOAc), yellow oily product: IR (KBr): $\overline{\nu}$ 2850, 2950, 1710, 1480, 1410, 1125, 1175, 1025, 700, 640 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.44-1.66 (m, 4H), 1.82-1.99 (m, 4H), 1.64 (q, 2H, J = 6.6 Hz); 2.77 (t, 2H, J = 8.0 Hz), 3.47 (t, 4H, J = 6.3 Hz), 3.6 (t, 2H, J = 6.7 Hz), 7.29 (t, 1H, J = 4.8 Hz), 7.61 (d, 1H, J = 1.7 Hz), 8.52 (d, 2H, J = 4.3 Hz) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 25.49, 26.67, 29.46, 29.52, 29.59, 30.90, 32.50, 45.02, 69.44, 123.31, 136.06, 137.29, 147.03, 149.69 ppm. LRMS (ESI) (*m/z*): 256.3 $[M+H]^+$.

Procedure for the Synthesis of 3-(3-((6-fluorohexyl)oxy) propyl)pyridine (7)

To a solution of compound **5** (1.0 equiv.) dissolved in CH_2Cl_2, cooled at -60°C, and under inert atmosphere, 1.2 equiv. of Diethylamino sulfur trifluoride (DAST) was added. This solution was stirred for 2 hours and cooled at -60°C. After that, it was allowed to warm to room temperature and stirred for 18 hours (overnight). After this period, 5 mL of water was added to quench the DAST excess. Then, the reaction mixture was washed with a diluted solution of $NaHCO_3$ (5 w/v %) and water. The organic layer was dried (Na_2SO_4), filtered, and evaporated under reduced pressure. The residue obtained was purified by column chromatography (SiO_2, EtOAc) to yield

pure compound **7**.

3-(3-((6-fluorohexyl)oxy)propyl)pyridine **7**: Yield 6%, *Rf* = 0.40 (EtOAc), yellow oily product: IR (KBr): $\overline{\nu}$ 2927, 2860, 1737, 1641, 1463, 1354, 1257, 1242, 1172, 1026, 974, 935, 908, 748, 713, 526, 459, 403 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.41-1.43 (m, 4H), 1.57-1.70 (m, 4H), 1.86-1.93 (m, 2H), 2.68-2.72 (t, 2H, J = 8.0 Hz), 3.39-3.43 (t, 4H, J = 8.0 Hz), 4.45 (dt, 2H, $^2J_{HF}$ = 48.0 Hz, $^3J_{HH}$ = 8.0 Hz), 7.19-7.23 (m, 1H), 7.50-7.52 (d, 1H, J = 8.0 Hz), 8.43-8.45 (m, 2H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 25.09, 25.87, 29.50, 29.62, 30.26, 30.96, 64.48, 70.84, 84.1 (d, J_1 = 162.0 Hz), 123.27, 135.89, 137.22, 147.31, 149.99 ppm.

In Vitro Schizonticidal Antiplasmodial Activity

Plasmodium falciparum chloroquine-resistant (W2) strain was maintained in continuous culture using human red blood cells in RPMI 1640 medium supplemented with human plasma.[10] Human red blood cells and human plasma were provided by the Foundation of Hemotherapy and Hemathology of Minas Gerais (Fundação Hemominas). Parasites were synchronized using sorbitol treatment,[11] and the parasitemias were evaluated microscopically with Giemsa solution-stained blood smears. Antiplasmodial activity was determined using an ELISA anti-HRPII assay.[12] Briefly, a 96-well plate was coated with infected red blood cells at 0.05% parasitemia and 1.5% hematocrit. Different concentrations of the compounds were added in triplicate, and twelve compound-free wells were used as controls (6 frozen after 24 hours as the HRPII background). After incubation (72 hours), the plate was frozen and thawed twice, and an ELISA using anti-HRPII antibodies was performed. The results were expressed as the mean of the half-maximal inhibitory dose (IC_{50}) of three assays with different drug concentrations performed in triplicate, compared with drug-free controls. Curve fitting was performed using OriginPro 8.0 software (Origin Lab. Corporation, Northampton, MA, USA).

In Vitro Cytotoxicity Test

The noncancerous human lung fibroblast cell line WI-26 VA4 (ATCC CCL-95.1) was used to assess cell viability after each chemical treatment employing the MTT colorimetric assay.[13] Briefly, 1×10^6 cells were plated in 96-well plates with RPMI 1640 medium supplemented with fetal bovine serum (FBS) and penicillin-streptomycin antibiotics. Then, plates were incubated overnight at 37 °C, 5% CO_2, followed by treatment with each compound solubilized in DMSO 0.1% (v/v). Negative control groups were constituted of cells without treatment. Five serial dilutions (1:10) were made from a stock solution (10 mg mL^{-1}) using RPMI supplemented with 1% FBS. After 48 hours of incubation, cell viability was evaluated by discarding the medium and adding 100 µL of MTT 5%, followed by 3 h of incubation. Then, the supernatant was discarded and the insoluble formazan product was dissolved in DMSO. The optical density (OD) of each well was measured using a microplate spectrophotometer at 550 nm. The OD in untreated control cells was defined as 100% cell viability. All assays were performed in triplicate. The selectivity index (SI) of the 3-APA analogs was calculated as: SI = IC_{50} WI-26 VA4/ IC_{50} *P. falciparum*.

Statistical Analysis

Analysis of variance (ANOVA) was performed followed by the Tukey-Kramer multiple comparisons post-test with a significance level of 0.05. Statistical analysis was performed using OriginPro 8.0 software (Origin Lab. Corporation, Northampton, MA, USA).

Heme Binding Experiments

Heme titration experiments were carried out in a UV-Vis spectrophotometer (Thermo Scientific, Model Genesys 10S) using 1 cm path cuvettes. *Preparation of Heme Stock Solution:* A 1.61 mM heme stock solution was prepared by dissolving 10.5 mg of hematin chloride in 1 mL of DMSO. *Preparation of Ligands Stock Solution:* Concentrated solutions of 22.78 mM, 131.0 mM, 57.2 mM, and 10.45 mM of ligand stock solution were prepared by dissolving 5.4 mg, 33.5 mg, 13.7 mg, and 10.0 mg of compounds **5, 6, 7,** and chloroquine, respectively, in 1 mL of DMSO. *Buffer:* A 0.020 M HEPES 40% DMSO buffer with an apparent pH value of 7.4 was prepared by adding 100 mL DMSO and 5 mL of 1 M HEPES, comprising a final volume of 250 mL of distilled water. The solution was stored at 4°C. *Titration Experiments:* 5 µM of heme solution was prepared from the concentrated stock solution (1.61 mM) using HEPES buffer. Solutions of 0.5 mM of ligands 5 and 6; 2.0 mmol L^{-1} of ligand 7 and chloroquine were prepared from concentrated solutions dissolved in 5 µM heme solution to avoid dilution effects. Titrations were performed by adding small volumes of the ligand solutions to a 5.0 µM heme solution, varying the concentrations of the ligands from 0 to 5.24 µM, from 0 to 200.9 µM, from 0 to 766.9 µM, and from 0 to 161.0 µM for compounds 5, 6, 7, and chloroquine, respectively. After each addition, the absorbance was measured from 300 nm to 500 nm. All experiments were performed in triplicate. Curve fitting was performed using GraphPad Prism 5.01 software.

Results

Chemistry

Compounds **6** and **7** were obtained in five steps from the available starting materials as shown in Scheme 1. The synthesis of compounds 1 to 5 was described previously.[14] In brief, 1,6-hexanediol 1 was selectively monoprotected to generate the corresponding monotetrahydropyranyl acetal 2. Compound 2 was then mesylated using traditional conditions, resulting in compound 3. Next, Williamson etherification was performed under phase-transfer catalysis of commercially available 3-(pyrid-3-yl) propan-1-ol with compound 3 to give compound 4. Then, a deprotection was performed using HCl, affording compound 5. Finally, the hydroxyl group of compound 5 was substituted by a chlorine and a fluorine atom using N-chlorosuccinimide and DAST to afford compounds 6 and 7, respectively.

To optimize the antiplasmodial activity of compound 5, analogs 6 and 7 were obtained through halogenation. These compounds had their *in vitro* growth inhibitory activity evaluated against a chloroquine resistant strain of *P. falciparum* (clone W2) with chloroquine as a positive control.

Additionally, both compounds were tested *in vitro* against the human cell line WI-26 VA4 (noncancerous human lung fibroblast cell line) to evaluate their cytotoxicity (techniques are described in Supporting Information, items 2 and 3).

As depicted in Table 1, halogenation by inserting chlorine and fluorine in compounds 6 and 7, respectively, was found to improve their antiplasmodial activity (7.2 μM and 8.3 μM, respectively) to about 25 times higher than that of compound 5 (210.7 μM). Interestingly, both compounds were less toxic to the human cell line than compound 5, which was demonstrated by an increase in the selectivity index (SI) from 1.9 (compound 5) to 4.5 (compound 6) and 35.1 (compound 7). The insertion of a fluorine atom in compound 7 led to a higher selectivity to *P. falciparum*.

Discussion

Improvement in biological activity as a result of halogenation has been used by the pharmaceutical industry and noted by other researchers during hit-to-lead or lead-to-drug conversions.[15, 16] The incorporation of halogenation atoms can improve metabolic stability,[17] enhance membrane permeability,[18] and favor molecular recognition between ligands and its receptors through the formation of halogen bonds.[19]

Moreover, as compounds 6 and 7 exhibited good *in vitro* activity against *P. falciparum,* they are candidates for further *in vivo* studies in the near future. The observation of the *in vivo* acute toxicity and efficacy data of these compounds is one more critical step to improving their likelihood of clinical success. This and other clinical data could support the development of a new antimalarial formulation to treat affected people.

Once the biological activity of compounds 6 and 7 was verified, the next step was to determine if halogenation altered the mechanism of action of the series. Inhibition of hematin polymerization was investigated by UV-Vis spectroscopy (Supporting Information, item 4). This method was chosen, because it can detect free hematin in submicromolar concentrations, as in the parasite's digestive vacuole.[20] An absorption band (Soret band) at 401 nm indicates the presence of free hematin, and a decrease in the intensity of the Soret band indicates the formation of a complex between hematin and the tested compound.[21] As shown in Figures 1a and 1b, compound **5** exhibited an interaction pattern with hematin similar to that of CQ.

UV-Vis titration experiments confirmed that the replacement of the hydroxyl group in compound 5 with chlorine or fluorine atoms (in compounds 6 and 7, respectively) did not alter the ability of these compounds to form a complex with hematin. As shown in Figures 1c and 1d, the Soret band intensity was decreased with compounds 6 and 7, indicating the formation of a complex with hematin. Similar results in the presence of antimalarial candidates have also been reported by other researchers.[22] Thus, compounds 5, 6, and 7 exhibited similar patterns of hematin binding (Figures 1b, 1c, and 1d, respectively). However, it is still not clear whether halogenation improves antiplasmodial activity by enhancing membrane permeability or molecular recognition.

All the data collected in this work suggests that compound 7 is a good starting point for further chemical optimization. However, according to Katsuno et al,[23] a compound to be assayed *in vivo* should fulfill specific requirements, such as IC_{50} <100 nM against *P. falciparum* strains and SI greater than 100 fold against mammalian cell lines.

Table 1. *In Vitro* Antiplasmodial Activities, Cytotoxicities, and Selectivity Indices of Chloroquine and Compounds **5**, **6**, and **7**

Compound	IC_{50} (μM) (mean ± SD[a])		SI[b]
	P. falciparum (W2)	WI-26 VA4	
5	210.7 ± 18.1	421.3 ± 12.7	1.9
6	7.2 ± 1.2*	32.3 ± 2.8*	4.5
7	8.3 ± 2.0*	291.7 ± 15.4*	35.1
CQ	0.4 ± 0.07*	> 100	>270

Research Highlights

What Is Already Known?

- Malaria is a disease that affects millions of people worldwide;
- The emergence of resistance to antimalarials is a great concern;
- Many attempts to discover new antimalarials has been made over the years.

What This Study Adds?

- 3-Alkylpyridine marine alkaloid (3-APA) analog corresponds to a new scaffold which has promising antimalarial activity;
- Halogenation is an interesting approach, because it leads to enhanced antimalarial activity and selectivity;
- Halogenated 3-APA analogs inhibited hemozoin formation, similar to the chloroquine mechanism of action.

Conclusion

A simple substitution of the hydroxyl group in a compound by a halogen atom can lead to significant changes in its antiplasmodial activity without altering its ability to bind to the target. However, it is still necessary to verify the effects of halogenation in these compounds; thus, further studies are necessary. As it has been shown before, this approach combined with reports of successful oriented synthesis and molecular hybridization could be an attractive alternative for the optimization of potential new antimalarial candidates. Therefore, further lead optimization of the halogenated compound 7 is required as it might identify new potential antimalarial candidates.

Authors' Contributions

Synthesis and structural elucidation: CdeSB and JdaCA; Performed the biological experiments: DSMG and CFAdeB; Biophysical heme binding study: RMRV and CdeSB; Coordinated the research: FdePV and GHRV. The manuscript was written with the contributions of all authors..

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Ethical Approval

Not applicable. The assays were not performed in humans or animals.

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