PROTOZOOLOGY - ORIGINAL PAPER

In silico evaluation and in vitro growth inhibition of *Plasmodium falciparum* by natural amides and synthetic analogs

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Abstract

Malaria, caused by protozoa of the genus *Plasmodium*, is a disease that infects hundreds of millions of people annually, causing an enormous social burden in many developing countries. Since current antimalarial drugs are starting to face resistance by the parasite, the development of new therapeutic options has been prompted. The enzyme *Plasmodium falciparum* enoyl-ACP reductase (P*f*ENR) has a determinant role in the fatty acid biosynthesis of this parasite and is absent in humans, making it an ideal target for new antimalarial drugs. In this sense, the present study aimed at evaluating the in silico binding affinity of natural and synthetic amides through molecular docking, in addition to their in vitro activity against *P. falciparum* by means of the SYBR Green Fluorescence Assay. The in vitro results revealed that the natural amide piplartine (**1a**) presented partial antiplasmodial activity (20.54 µM), whereas its synthetic derivatives (**1m**—IC$_{50}$ 104.45 µM), (**1b**, **1g**, **1k**, and **14f**) and the natural amide piperine (**18a**) were shown to be inactive (IC$_{50}$ > 200 µM). The in silico physicochemical analyses demonstrated that compounds **1m** and **14f** violated the Lipinski's rule of five. The in silico analyses showed that **14f** presented the best binding affinity (− 13.047 kcal/mol) to P*f*ENR and was also superior to the reference inhibitor triclosan (− 7.806 kcal/mol). In conclusion, we found that the structural modifications in **1a** caused a significant decrease in antiplasmodial activity. Therefore, new modifications are encouraged in order to improve the activity observed.

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Introduction

Malaria is a disease that constitutes a serious public health problem worldwide, with an estimated 219 million cases in 2017 (WHO 2018). The disease is caused by protozoa of the genus *Plasmodium*, of which five species are known to cause disease in humans (Cox 2010; Amir et al. 2018). *Plasmodium falciparum*, alongside *Plasmodium vivax*, are the species that cause most of the cases and deaths annually, making them the most relevant species both clinically and epidemiologically (Hay et al. 2010). These species can cause severe form of malaria in some cases (Mackintosh et al. 2004; Alexandre et al. 2010). For *P. falciparum*, this severe form may cause serious organ damage, resulting in respiratory and neurological problems, in addition to fetal loss in pregnant women (Mackintosh et al. 2004). For *P. vivax*, common clinical manifestations are

thrombocytopenia, and renal, hepatic, cerebral, and pulmonary involvement (Zubairi et al. 2013).

The chemotherapy currently used in the treatment of malaria, which is based on artemisinin combination therapy (ACT), has started to face resistance by the parasite in some regions of the globe, especially in Southeast Asia (Ashley et al. 2014), which prompts the development of drugs targeting specific pathways of the parasite.

In this sense, dissociative or type II fatty acid synthase, named FAS II, constitutes a promising molecular target for potential antimalarial drug candidates, since it is absent in humans (Qidwai and Khan 2012). *Plasmodium falciparum enoyl-ACP reductase* (*Pf*ENR), which belongs to this pathway, is involved in the final step of fatty acid chain elongation, catalyzing the reduction of enoyl-ACP to acyl-ACP using NADH as an electron donor, and is also the rate-limiting enzyme in this cycle (Tasdemir 2006).

Regarding potential compounds for antiparasitic agents, natural products derived from plant secondary metabolism possess a plethora of potential antiparasitic molecules (Wink 2012). With respect to antimalarial therapy, the alkaloid quinine originated a series of analogs of great relevance for the treatment of disease (Krafts et al. 2012). In this context, plants that are rich in alkaloids—which encompasses the species of the genus *Piper* (Gutiérrez et al. 2013)—constitute a great potential to develop new compounds against the disease. Piplartine and piperidine, two alkaloids isolated from *Piper* species, have been shown to present various biological activities. Piplartine presents activity against various parasitic species (*Leishmania amazonensis* Lainson & Shaw, 1972; *Leishmania infantum* Cunha & Chagas, 1937; *Leishmania donovani* Laveran & Mesnil, 1902 and *P. falciparum*), especially *Trypanosoma cruzi* Chagas, 1909 (Araújo-Vilges et al. 2017; Bodiwala et al. 2007; Moreira et al. 2018; Cotinguiba et al. 2009; Gomes et al. 2014; Silva-Jardim et al. 2004). In this light, this study aimed at evaluating the in silico binding affinity of two natural amides and five synthetic analogs with *Pf*ENR, their in silico physicochemical properties, and their in vitro antiplasmodial activity.

Materials and methods

All the compounds were obtained using the following methodology

Isolation of the natural compounds (piplartine and piperine)

Roots of *Piper tuberculatum* Jacq, 1897 were harvested on the University of São Paulo (USP) Campus, São Paulo, Brazil. A voucher specimen (Kato-0169) has been deposited at the Institute of Biosciences Herbarium. For the isolation of piplartine, 100 g of ground roots of *P. tuberculatum* were

extracted with a mixture of methanol/chloroform 1:2 (1 L) for 72 h. The mixture was rotary evaporated under reduced pressure to yield a white solid which, after recrystallization in MeOH, produced pure piplartine (**1a**) (PubChem CID: 637858), which was identified by comparison of established NMR and MS data with reported data (Araújo-Vilges et al. 2017).

Fruits of *Piper nigrum* Koehler, 1887 were purchased from a local market in São Paulo. Piperine (**18a**) was purified from 250 g of dried *P. nigrum* fruits. The fruits were ground into a powder and extracted twice with 2 L of methanol/chloroform (1:2). The extract was filtered and concentrated in a rotary vacuum evaporator. The crude extract was subjected to silica column chromatography eluted with hexanes/ethyl acetate mixtures of increasing polarity. Fractions containing piperine (**18a**) were pooled and recrystallized in methanol yielding a yellow crystal. Piperine (**18a**) (PubChem CID 638024) was identified by NMR and MS analyses and comparison with reported data (Parmar et al. 1997; Araújo-Júnior et al. 1997).

Synthesis of the synthetic analogs

Compound **1b** (PubChem CID: 53440296) was obtained by the catalytic hydrogenation (4 atm of hydrogen, Pd–C) of piplartine (**1a**) for 4 h and was identified by comparison of NMR and MS analyses with reported data (Fokoue et al. 2018).

Compounds **1g** and **1k** were synthesized by adding triethylamine (3 equivalents) and the appropriate amines (i.e., pyrrolidine and n-pentylamine) to a methylene chloride solution of various acyl chlorides (1.0 equivalent). The reaction mixtures were stirred overnight at room temperature, quenched with a saturated aqueous ammonium chloride solution and then extracted with methylene chloride 3 times. Combined organic phases were washed with brine and dried over anhydrous magnesium sulfate. After filtration and concentration, the residues were purified by flash chromatography over a silica gel using hexanes-ethyl acetate (typically 30–50%) as an eluent, yielding the desired amides. Compounds **1g** (PubChem CID: 121169) and **1k** (PubChem 19173974) were identified by comparison of NMR and MS analyses with reported data (Campelo et al. 2018; Fokoue et al. 2018).

Compounds **1m** and **14f** were synthesized by adding 2*E*-3,4,5-trimethoxycinnamic acid and 2*E*-3,4-methylenedioxycinnamic acid (1 equivalent) to a solution of THF 0.9 equivalent of *N*,*N*′-dicyclohexylcarbodiimide (DCC). The reaction mixture was stirred overnight at room temperature and dried on a rotary evaporator to remove any remaining solvent. The product was dissolved in dichloromethane (DCM), a saturated solution of NaHCO$_3$ was added, and the resulting solution was washed three times with DCM. The organic phase was combined, dried with MgSO$_4$, filtered, and concentrated under pressure. The obtained crystalline

product was then recrystallized on hot methanol or purified by chromatography over silica-gel using hexanes-ethyl acetate (typically 30–50%). Compound **1m** was identified by comparison of NMR and MS analyses with reported data (Campelo et al. 2018).

Compound **14f** was characterized according to the following data: 3-[(2E)-3-(2H-1,3-benzodioxol-5-yl)prop-2-enoyl]-1,3-dicyclohexylurea (Fokoue 2015).

^{1}H NMR (300 MHz; CDCl$_3$): δ 7.57 (d; J = 15.0 Hz; 1H, H2); 7.13 (sl, NH); 6.97 (dd; J = 8.5 and 1.5 Hz; 1H, H9); 6.95 (d; J = 1.5 Hz; 1H, H5); 6.80 (d; J = 8.0 Hz; 1H, H8); 6.55 (d; J = 15.0 Hz; 1H, H3); 6.01 (s, 2H, OCH$_2$O); 4.13–4.06 (m; 1H, H1a); 3.78–3.73 (m; 1H, H1b); 1.99–1.19 (m; 20H, H2a, H2b, H3a, H3b, H4a, H4b, H5a, H5b, H6a, H6b). **^{13}C NMR** (75 MHz; CDCl$_3$): δ 166.64 (C1); 154.07 (C*); 149.34 (C6); 148.23 (C7); 143.14 (C3); 129.11 (C4); 124.20 (C9); 117.26 (C2); 108.51 (C8); 106.28 (C5); 101.51 (OCH$_2$O); 55.90 (C1a); 49.92 (C1b); 34.90 (C2b); 33.95 (C6b); 32.78 (C2a); 30.92 (C6a); 26.25 (C4b); 25.61 (C4a); 25.45 (C3b); 25.37 (C5b); 24.93 (C3a); 24.71 (C5a). **EI-MS (m/z)** (%): 273 (35). 257 (25). 241 (25). 190 (90). 175 (55). 145 (75). 118 (60). 98 (72). 89 (100). 63 (35). 44 (50). **ESI-MS**: calculated for $C_{23}H_{31}N_2O_4^+$ [M + H]$^+$: 399.2278; found: 399.2210. **IR (KBr, v$_{máx}$/cm^{-1})**: 3256. 3047. 2931. 2855. 2118. 1704. 1646. 1542. 1503. 1491. 1448. 1388. 1344. 1255. 1229. 1042. 989. 936. 810.

Virtual screening

OSIRIS property explorer and Molinspiration

Initially, a virtual screening of the natural compounds and their derivatives was performed. This analysis allows for a rational choice of drugs by selecting those that present adequate physicochemical properties, which might, in turn, translate into good oral bioavailability. For these analyses, we used the software OSIRIS property explorer (https://www.organic-chemistry.org/prog/peo/) and the server Molinspiration (http://www.molinspiration.com).

OSIRIS

The substances were drawn in the software OSIRIS property explorer (https://www.organic-chemistry.org/prog/peo/) and compared with a list of approximately 5300 different fragments in respect to their *druglikeness* properties. These fragments are obtained from 3300 commercial drugs and 15,000 chemicals available from the company Fluka®.

The software OSIRIS computes theoretical physicochemical and toxicological properties of compounds, aiming to evaluate some properties and toxicological assessments such as mutagenic, tumorigenic, irritant, and reproductive risks. The toxicological results are color-coded, wherein red indicates

high risk, yellow indicates moderate risk, and green indicates no risk. The software also evaluated the *druglikeness* and *drugscore* of the respective compounds, which aim to measure whether or not a compound qualifies as a drug by considering its physicochemical properties, structural similarity with commercially available drugs, and toxicity risks.

The software also shows the *druglikeness*, a parameter that evaluates how structurally similar the compound is to commercially available drugs. Its values range from negative to positive values, with positive values indicating that the compound is structurally similar to commercially available drugs, whereas negative values indicate otherwise. Finally, the software calculates the *drugscore*, a final parameter which is based on (1) pharmacokinetic and physicochemical features, (2) toxicological prediction, and (3) *druglikeness*. The *drugscore* values range from 0 to 1, with values close to 1 indicating that the compound may qualify as a drug (https://www.organic-chemistry.org/prog/peo/).

Molinspiration

The evaluated compounds were also analyzed in the server Molinspiration, which calculated the number of violations to the Lipinski's rule of 5 according to their: molecular weight (≤ 500 μM), clogP (≤ 5), hydrogen bond acceptors (≤ 10), and hydrogen bond donors (≤ 5) (Lipinski 2004). The server also evaluated two additional properties: the total polar surface area (TPSA) and the number of rotatable bonds. According to Veber et al. (2002), it is desirable that drug candidates should have a TPSA ≤ 140 Å^2 as well as a number of rotatable bonds ≤ 10.

Molecular modeling and molecular docking studies

The molecular modeling study was conducted in partnership with the Laboratory of Molecular Modeling and Computational Simulation of the Federal University of Alfenas – Minas Gerais, Brazil. All computer applications were run on OpenSUS Tumbleweed. Structures of the ligands **1a**, **1b**, **1g**, **1k**, **1m**, **14f**, **18a**, and triclosan (ligand used in the redocking analysis) were constructed using the software Maestro 10.2.010. The software LigPrep 3.4 with OPLS_3 force field and ionization state for pH 7.0 ± 2.0 was used to prepare the ligands involved in the studies. The crystallographic structures of P*f*ENR with triclosan (PDB ID: 1UH5) from *P. falciparum* were obtained from the Protein Data Bank (PDB) database and the software Protein Preparation Wizard was used for the preparation of these receptors. Co-crystallized NAD and triclosan present in 1UH5 were removed from the structure and chain A alone was selected for docking studies. The OPLS3 force field in MacroModel 9.9 was used for optimization. Molecular docking studies between P*f*ENR and the ligands were performed using the

Induced Fit Docking protocol, in which the program Prime was used for refinement of the compounds and the program Glide provided the scoring considering the proteins and the flexible ligand. The grid box area was defined with the amino acids Tyr 277, Ile 323 and Phe 368. All computer programs used belong to the Schrödinger suite (Pidugu et al. 2004; Schrödinger, Small-Molecule Drug Discovery Suite 2015; Schrödinger release 2015a, b, c).

In vitro culture of P. falciparum

Erythrocytic forms of the W2 strain of *P. falciparum* were used in the antiplasmodial assays. The parasites were cultured in O+ red blood cells in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 300 μM hypoxanthine, 11 mM glucose, 40 μg/ml gentamicin 10% (*v/v*), and O+ plasma or 5% albumax (Gibco) under the conditions established by Trager and Jensen (1976). The erythrocytes added to the culture originated from a single volunteer donor, with a final hematocrit of 4%. The culture flasks were kept in an incubator at 37 °C, with the addition of a composite gaseous mixture (5% CO_2, 5% O_2, and 90% N_2). The medium was changed every 48 h. The project was approved by the local Ethics and Research Committee (CEP) under number: 83791418.8.0000.5300, case: 2.541.143.

Determination of parasite growth inhibition (IC$_{50}$)

The substances were diluted in dimethylsulfoxide (DMSO), at concentrations up to 0.5%. The serial dilution started from an initial concentration of 2000 μM in the first well (20 μL). Cultures were synchronized until they reached the desired parasitemia of 8% young trophozoites, as described by Lambros and Vanderberg (1979). Subsequently, the parasite culture with a predominance of ring forms was adjusted to 0.5% parasitemia and 2% hematocrit. Thus, all compounds were diluted 10×, at concentrations from 200 to 1.56 μM. Three controls were used: artemisinin (170 nM) was used as the positive control, untreated red blood cells infected with *P. falciparum* constituted the negative control, and uninfected red blood cells were used as a blank (whose fluorescence was discounted from all values obtained).

Plates were incubated at 37 °C with 5% CO_2 for 48 h. Thereafter, the supernatant was discarded without suspending the red cells. To the latter, 1× PBS was added (200 μL/well) and centrifuged at 1500 rpm for 10 min. In parallel, 200 μL of PBS 1× was added to a flat-bottom 96-well culture plate for fluorescence reading. After centrifugation of the plate, the supernatant was discarded again. In the plate containing the culture, 200 μL of lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.08% Triton X-100, 0.008% saponin in 1× PBS, pH 7.5) containing SYBR Green I (Invitrogen) was added in the following ratio: 2 μL of SYBR Green I dye to 10 ml of the

lysis buffer solution. The mixture was homogenized and 200 μL of it was transferred to the reading plate where 200 μL of PBS 1× was previously added. The plate was incubated for 30 min at room temperature. Subsequently, a UV/visible spectrophotometer reading (Synergy HT (BioTek)) with an excitation of 485 nm and emission of 535 nm was performed. The concentration at which the compounds caused 50% of parasite growth inhibition (IC$_{50}$) was determined by the dose-response curve of the triplicate mean for each evaluated compound (Smilkstein et al. 2004).

Cultivation of mammal cell lines

VERO cells (renal cells isolated from the kidneys of the African monkey *Cercopithecus aethiops*) and HepG2 cells (cell line derived from human hepatocarcinoma) were used for the cytotoxicity assays. These cell lines are related to the excretion of substances and to drug metabolism, respectively. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Gibico) and 40 mg/L gentamicin (complete medium) in an incubator with 5% CO_2 at 37 °C. The culture was monitored daily by optical microscopy, and the medium was changed whenever necessary until 90% confluence was reached, indicating favorable conditions for the cytotoxicity assay (Calvo-Calle et al. 1994).

Cytotoxicity assay using the MTT method

After reaching the desired confluence, the mammal cell lines were detached from the bottom of the flasks with the aid of trypsin 1× (Sigma-Aldrich) and centrifuged (1500 rpm for 10 min). The supernatant was removed and the pellet resuspended in 1 ml of complete medium. An aliquot of 10 μL of this volume was removed so that the cells could be counted in a Neubauer chamber using an optical microscope. Afterwards, the culture was adjusted to 1×10^4 cells/well. The cells were then plated in 96-well plates and incubated for 24 h at 36 °C. All compounds were distributed separately on the plate, in triplicate, using the serial dilution method (200 to 1.56 μM). The culture was incubated again (48 h). Untreated cells were constituted as the positive control, whereas the cells treated with lysis buffer containing Tris at 20 mM (Sigma-Aldrich), EDTA at 5 mM (Dinâmica), saponin at 0.008%, and Triton X-100 at 0.008% (*v/v*) (Sigma-Aldrich) constituted the negative control.

After the treatment period, the plates were revealed using the MTT technique ([3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide]) (Mossmann 1983). This technique evaluates cell viability through mitochondrial dehydrogenase, present only in metabolically viable cells. After the addition of MTT (Sigma-Aldrich) at a concentration of 5 mg/ml, the plate was incubated for 4 h, the supernatant discarded immediately, and 100 μL of dimethyl sulfoxide (DMSO) (Dynamic) was added to dissolve the formazan

crystals (violet coloration). A spectrophotometer (Biochrom, Asys Expert Plus) was used to read the absorbance (570 nm) of the test plate. Afterwards, the software Origin 9.1 (Origin Lab Corporation, Northampton, MA, USA) was used to determine the cytotoxic concentration for 50% of the cell population (CC_{50}).

Selectivity index (SI)

The SI of the evaluated compounds was obtained using the ratio: CC_{50}/IC_{50}. Compounds whose SI were ≥ 10 were considered to be selective, whereas compounds with SI < 10 were considered non-selective (Katsuno et al. 2015; Gomes et al. 2014; Nogueira and Rosário 2010).

Results and discussion

Piplartine, piperine, and analogs

For the present study, five analogs (**1b**, **1g**, **1k**, **1m**, and **14f**) were synthesized from the natural amide piplartine following previously published literature, without major changes, and characterized by spectrometric and spectroscopic data. The yields were not optimized since we focused on obtaining a sufficient amount of pure compounds for the assays.

In silico prediction of physicochemical and toxicological properties of the evaluated compounds.

Due to the importance of some physicochemical features for the biological activity of chemicals (Lipinski 2004), the virtual prediction of parameters such as molecular weight, hydrogen bond acceptors and donors and cLogP (computed partition coefficient), as well as possible biological activities (*drugscore*), were calculated using the software OSIRIS Property explorer and the server Molinspiration.

Most of the compounds in Tables 1 and 2 are within the limits established by Lipinski's rule of five, with some observations. Compounds **1m** and **14f** presented a red alert for mutagenicity and reproductive toxicity (the latter was also presented by compounds **14f** and **18a**), which can be explained by the presence of groups with a large volume in their structures. Compounds **1m** and **14f** presented one violation to the Lipinski's rule of five. It is important to stress, however, that these alerts do not necessarily exclude compounds **14f** and **18a** from being safe drugs, but rather indicate a probable risk.

The molecular weights of the compounds averaged 303.36 ± 58.15 g/mol; the cLogP values averaged 2.87 ± 1.09 and were below 5 for all compounds. This parameter is related to lipophilicity, in that compounds with cLogP values < 5 present inadequate oral bioavailability, owing to the difficulty of passing through biological membranes (Oliveira 2013).

According to Freitas (2015), high and positive cLogP values indicate a hydrophobic and lipophilic nature of the compounds, while negative values indicate a hydrophilic nature. Compounds **14f** and **1m** presented cLogP values = 4.7 and 4.38, respectively, indicating that these molecules are hydrophobic/lipophilic. The cLogP of compounds **1a** and **18a** were = 1.78 and 3.6, which means that these compounds are hydrophobic/lipophilic. The compounds also presented desirable values for *druglikeness* (0.115 ± 3.30), *drugscore* (0.385 ± 0.19), and *solubility* (-3.04 ± 1.35). The *drugscore* values can range from 0 to 1 (a compound that completely qualifies as a drug). The positive values of *druglikeness* observed mean that the evaluated compounds possess fragments that are commonly found in commercially available drugs (Magalhães 2009).

The S (solubility—Table 1) scale classifies the compounds' solubility in water as being insoluble for values lower than 10; slightly soluble < 6; moderately soluble < 4; soluble < 2; very soluble < 0 (Guerra 2019). The S (solubility) values for the natural compounds 1a (piplartine) and 18a (piperine) were 2.39 and -3.61, respectively. These results underpin their hydrophobic/lipophilic attributes. Since they are poorly soluble in water, these compounds have limited biological applicability. Aqueous solubility has a determinant role in the absorption and distribution process to reach the blood circulation. Most commercial compounds have S (mol/L) greater than -4.00 (OSIRIS Property explorer). In general, poorly water-soluble compounds are incompletely absorbed in the body. However, water solubility can be increased and/or improved by the addition of hydrochlorides, sodium salts, etc.

The total polar surface area (TPSA), hydrogen bond donors, hydrogen bond acceptors and rotatable bonds were within the desired thresholds for all compounds in Table 2, except **14f**, which presented more than five hydrogen bond donors. Compounds **1g** and **1a** presented the best *druglikeness* and *drugscores* (Tables 1 and 2), indicating that these compounds are the ones that best qualify as drugs. Therefore, we infer that the modifications made at the dihydropiperidinone moiety of the ring of compound **1a**, substituted by a piperidinyl ring **(1g)** contributed to these results.

Inhibitory concentration (IC_{50}) and cytotoxic concentration (CC_{50})

The compounds **1a** and **1m** obtained $IC_{50} = 20.54$ μM and 104.45 μM respectively. The compounds **1b**, **1g**, **1k**, **14f**, and **18a** obtained IC_{50} values > 200 μM (Table 3). The control with the solvent DMSO did not affect cell viability.

The cytotoxicity assays demonstrated that piplartine was toxic on the evaluated mammal cell lines and on *P. falciparum*, which suggests that the compound might be exerting its effects through non-specific mechanisms, involving enzymes, receptors and/or pathways that are present in both protozoa and mammal cells. Some studies show that the biological and toxicological activity of piplartine may be

Table 1 Physicochemical properties, *druglikeness* and toxicity alert of compounds **1a**, **1b**, **1g**, **1k**, **1m**, **14f**, and **18a**

Compounds	MW	Toxicity alert				Drug-score			
		MUT	TUM	IRRI	REP	CP	S	DL	DS
1a	317.34	🟩	🟩	🟩	🟩	1.78	-2.39	3.8	0.39
1b	321.37	🟩	🟩	🟩	🟩	2.17	-2.49	2.89	0.14
1g	291.35	🟩	🟩	🟩	🟩	2.28	-2.37	5.41	0.72
1k	307.39	🟩	🟩	🟩	🟩	2.99	-3.16	-2.77	0.44
1m	444.57	🟥	🟩	🟩	🟩	4.38	-5.29	0.45	0.24
14f	398.50	🟥	🟩	🟩	🟥	4.7	-5.95	-2.42	0.09
18a	285.34	🟩	🟩	🟩	🟥	3.6	-3.61	0.6	0.39

🟩 : non-toxic; 🟨 : slightly toxic; 🟥 : toxic

MUT mutagenic, *TUM* tumorigenic, *IRRI* irritant, *REP* reproductive, *CP* cLogP, *S* solubility, *DL* druglikeness, *DS* drug-score.

attributed to the presence of α,β-unsaturated carbonyl groups, in which the substitution of any of these unsaturated groups led to a loss of biological activity as well as a decrease in cytotoxicity (Campelo et al. 2018; Bezerra et al. 2013; Bezerra et al. 2008; Ponte-Sucre et al. 2015).

Selectivity Indexes were not obtained for the compounds **1b**, **1g**, **1k**, **14f**, and **18a**, since neither the CC_{50} nor the IC_{50} values could be determined for these compounds.

Molecular docking studies on a series of molecules against *P. falciparum* P *f*ENR

The molecular docking results revealed that the compounds that presented the highest binding affinities for the enzyme were **1m** and **14f** (Supplementary material). However, the rest of the evaluated compounds also obtained binding energies that were higher than the reference inhibitor triclosan.

All the evaluated compounds presented superior affinity values for *Pf*ENR (GScore (Glide Score)) when compared to the reference inhibitor triclosan. This might be explained by the higher volume of the compounds when compared to triclosan, along with the contribution of steric effects, thus

Table 2 Physicochemical properties of compounds **1a**, **1b**, **1g**, **1k**, **1m**, **14f**, and **18a**

Physicochemical properties

Compounds	TPSA	HBA	HBD	VIO	ROT	VOL
1a	65.08	6	0	0	5	289.03
1b	65.08	6	0	0	6	301.40
1g	48.01	5	0	0	5	276.23
1k	54.80	5	1	0	9	303.25
1m	77.11	7	1	1	7	431.24
14f	67.88	4	6	1	4	378.53
18a	38.78	4	0	0	3	267.74

TPSA total polar surface area, *HBA* hydrogen bond acceptors, *HBD* hydrogen bond donors, *VIO* number of violations, *ROT* rotatable bonds, *VOL* volume

Table 3 Values of growth inhibition (IC_{50}), cytotoxic concentration (CC_{50}), and selectivity index (IS) of the evaluated compounds

Compounds	IC_{50} (μM)	CC_{50}			
		HepG2 (μM)	VERO (μM)	IS(a)	IS(b)
1a	20.54	14.98	23.37	0.73	1.14
1b	>200	>500	>500	–	–
1g	>200	>500	>500	–	–
1k	>200	>500	>500	–	–
1m	104.45	>500	>500	> 4.79	> 4.79
14f	>200	>500	>500	–	–
18a	>200	>500	>500	–	–

IS(a) selectivity index in relation to HepG2 cells, IS(b) selectivity index in relation to VERO cells

Table 4 Affinity values between the ligands and the enzyme P*f*ENR (*GScore*), number of hydrogen bonds (Hbond), and van der Waals (*good vdW*) interactions between the ligands and P*f*ENR (Schrödinger Suite, Induced Fit Docking Program)

Ligands	GScore (kcal mol^{-1})	Hbond	Amino acids that form hydrogen bonds	Good vdW
1a	− 10.762	1	Tyr277	404
1b	− 8.794	2	Tyr277, Lys285	334
1g	− 8.229	2	Lys285, Leu315	310
1k	− 9.659	3	Tyr111 (2), Tyr277	440
1m	− 10.981	1	Ala319	546
14f	− 13.047	2	Tyr111, Tyr277	483
18a	− 9.900	2	Ala219, Tyr277	313
Triclosan	− 7.806	0	–	183

increasing their affinity for the enzyme, which indicates that the molecules may present higher inhibitory activities than the reference compound (Table 4).

The compound **14f** (Table 4) presented the highest affinity value (GScore = − 13.047 kcal mol^{-1}), with 2 interactions per hydrogen bond (HBond) between the carbonyl oxygen atoms and the Tyr111 and Tyr277 residues, in addition to displaying a high number of favorable van der Waals (vdW) contacts (483). No parasite growth inhibition was observed for the compound **14f** at 200 μM, which might be explained by steric effects relative to the molecule's size or other physicochemical properties.

The molecule with the second highest binding affinity was **1m** (− 10.981 kcal mol^{-1}) (Table 4), also presenting a hydrogen bond interaction with the residue Ala319 and a superior number of favorable vdW contacts (546) when compared to **14f**. These data indicate that trimethoxyphenyl groups favor more hydrophobic interactions. The compound **1m** presented the second best IC$_{50}$ value (104.45 μM), which may be related to its molecular volume and to its access to the enzyme's active site. Other features that could possibly explain such differences between **1m** and **14f** are the number of hydrogen bond acceptors and rotatable bonds, which can influence the flexibility of the molecule and consequently, its interaction with the enzyme's active site (Veber 2002).

The compound that presented the third highest GScore value was **1a** (− 10.762 kcal mol^{-1}) (Table 4), with the presence of one hydrogen bond with the Tyr277 residue and 404 favorable vdW contacts, which are related to the small size of this molecule when compared to the derivatives **14f** and **1m**. Despite the satisfactory binding affinity values of these two compounds, they did not display in vitro antiplasmodial activity.

When comparing the in vitro IC$_{50}$ values, it was observed that **1a** presented the best results (20.54 μM) against *P. falciparum* owing to its molecular volume, which may facilitate its access to the active site of the enzyme. Regarding the in silico results displayed in Table 1, compound **1a**

presented a completely different hydrophobicity value (cLogP) when compared to the analogs. All structural modifications performed in **1a** were shown to substantially decrease its antiplasmodial activity. Fokoue (2015) reported that the solubility of drugs in a hydrophobic medium decreases their biological activities, which was observed in the present study for the compound's analogs.

It is important to stress that the IC$_{50}$ values of **1a** and **1k** against *P. falciparum* were previously reported in a study by Araújo-Vilges et al. (2017), being 19.5 μM and 79.1 μM, respectively (48 h of incubation). The strain and the culture used in the study are different from those of the present study, which might explain the differences between the results found in each study.

Gomes et al. (2014) when citing Ryckmans et al. (2009) state that it is desirable that the compounds evaluated for antiplasmodial activity present low IC$_{50}$ values in in vitro assays. In this perspective, Boechat et al. (2014) consider compounds with IC$_{50}$ < 10 μM to be active; with IC$_{50}$ > 10 and < 50 μM to be partially active; and compounds with IC$_{50}$ > 50 μM to be inactive. It is also noteworthy that substances with high molecular weights and high lipophilicity are more likely to present inadequate oral bioavailability. These features may minimize the risk for lack of specificity of the drug for its respective target, besides enabling one to administer lower doses, thus reducing the occurrence of adverse effects (Lipinski 2004).

The structural modifications of piplartine (**1a**) were performed at the dihydropiridinone moiety, generating the compounds **1b**, **1g**, **1k**, and **1m**. These alterations caused changes in the physicochemical properties of the in silico study, as well as in the in vitro growth inhibition assay against *P. falciparum*, rendering the molecules inactive (according to Boechat et al. 2014) against the parasite at concentrations up to 200 μM when compared to the natural compound piplartine (**1a**). In **14f**, the modifications occurred both at the dihydropiridinone and trimethoxybenzene moieties, which significantly altered its antiplasmodial activity and physicochemical properties.

Significantly, in the molecular docking studies, only affinity values were observed, i.e., whether or not energetically favorable interactions (electronic and steric effects) between ligand and enzyme were formed. Favorable affinity values may lead to intrinsic activity by the molecule, wherein the intrinsic activity depends on the affinity. The opposite, however, is not true: one can find favorable affinity values without the desired biological activity, since this activity is also related to other factors, such as bioavailability, metabolic stability, and others (Piccirillo and Amaral 2018).

It is important to highlight that the theoretical data (in silico) that present some violation do not eliminate the possibility of being evaluated in in vitro or in vivo tests; they only indicate which substances are valid and can guide investigators on whether or not to proceed with experimental analyses. In relation to toxicity characteristics (mutagenic, tumorigenic, genotoxic, etc.), there are in vitro and in vivo tests that allow for the evaluation of these properties.

This study reported for the first time the in silico affinity of the investigated amides against *Pf*ENR. The compounds obtained higher binding affinities than the known ligand triclosan, among which **1m** and **14f** presented the highest binding affinities.

However, the modifications caused a significant decrease in in vitro antiplasmodial activity in all analogs and even caused one compound (14f) to generate a toxicological alert. Therefore, new modifications on 1a are prompted in a continuous attempt to enhance its antiplasmodial activity and/or toxicity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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