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BIOMEDICAL SCIENCES

In silico ADMET prediction, evaluation of cytotoxicity in mouse splenocytes and preliminary evaluation of *in vitro* antimalarial activity of 4-(4-chlorophenyl) thiazole compounds

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Abstract: In this work, an *in silico* study and evaluation of the cytotoxicity of 4-(4-chlorophenyl)thiazole compounds against mouse splenocytes and the chloroquine-sensitive *Plasmodium falciparum* 3D7 strain are reported. The *in silico* results showed that the compounds have important pharmacokinetic properties for compounds with potential drug candidates. Regarding cytotoxicity assays against splenocytes, the compounds have low cytotoxicity. In addition, they were able to promote activation of these cells by increasing nitric oxide production without promoting cell death. Finally, they were able to promote cell proliferation. Regarding the *in vitro* anti-*P. falciparum* activity assays, it was observed that the compounds were able to inhibit the parasite's growth, presenting IC_{50} values ranging from 0.79 to greater than 10 μM. These results are promising when compared to chloroquine. Therefore, this study showed that 4-(4-chlorophenyl)thiazole compounds are promising candidates for antimalarials.

Key words: thiazoles, ADMET, mammalian cell cytotoxicity, antimalarial activity.

INTRODUCTION

Malaria is an infectious, systemic and parasitic disease caused by a unicellular protozoan of the genus *Plasmodium*. It is a common and life-threatening disease in many tropical and subtropical areas (Galinski 2022, Gujjari et al. 2022). Among the most important species of the genus, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. Knowlesi* stand out. However, *P. falciparum* is known to remain the most lethal species, followed by *P. vivax* (Gujjari et al. 2022). These parasites have an extremely complex heteroxenous life cycle, involving different hosts, such as humans and *Anopheles* mosquitoes (Galinski 2022).

Epidemiological data demonstrate that malaria cases are more prevalent in Africa, Asia and the Americas (Galinski 2022, Gujjari et al. 2022). An epidemiological update by the World Health Organization (WHO) published in 2022 showed that in 2021 there were an average of 619,000 deaths from malaria worldwide compared to 625,000 in the first year of the pandemic. In 2019, before the start of the pandemic, the death toll was 568,000. Furthermore, it was observed that malaria cases continued to increase between the years 2020 and 2021, but at a slower pace than in the period 2019 to 2020. The global malaria case count reached 247 million in 2021

compared to 245 million in 2020 and 232 million in 2019 (WHO 2022).

In this alarming context, the need for early diagnosis and treatment is evident, in addition to eliminating the mosquito vectors responsible for transmitting the parasitosis (Daily et al. 2022). However, several reports indicate the existence of strains of *Plasmodium* and *Anopheles* resistant to other antimalarials and specific insecticides still used today, respectively (Plowe 2022).

It is known that there are several drugs used in the treatment of malaria, such as quinine, quinolines, sulfones, pyrimethamine, artemisinin, mefloquine, chloroquine and antibiotics, all known for their ineffectiveness in the face of resistance quickly created by the *P. falciparum* species (Plowe 2022). In addition to the problems mentioned above, malaria control is even more undervalued due to the lack of an effective vaccine for this parasitosis (Galinski 2022, Gujjari et al. 2022).

Monotherapy may lead to parasite resistance more quickly. Therefore, a promising alternative has been the use of therapeutic combinations combining two or more compounds (Visser et al. 2022). However, the search for new antimalarial compounds becomes even more evident and necessary. Some compounds, then, have shown importance due to their therapeutic activity evaluated through several studies using strains of the genus *Plasmodium* (Santos et al. 2023). In this context, thiazoles stand out, since their main characteristics are versatility, high yield and high stability in different reaction conditions, as well as their wide applicability as intermediates of several important nuclei. In addition to promoting different biological activities (Ali & Sayed 2021).

Thus, in view of all the difficulties pointed out and commented on in relation to malaria. This study aimed to carry out an *in silico* study in order to predict the pharmacokinetic properties of 4-(4-chlorophenyl)thiazole compounds. In addition to evaluating the cytotoxic effects of the compounds against mouse splenocytes and the strain *P. falciparum* 3D7 sensitive to chloroquine. In order to contribute with new therapies for the treatment of malaria.

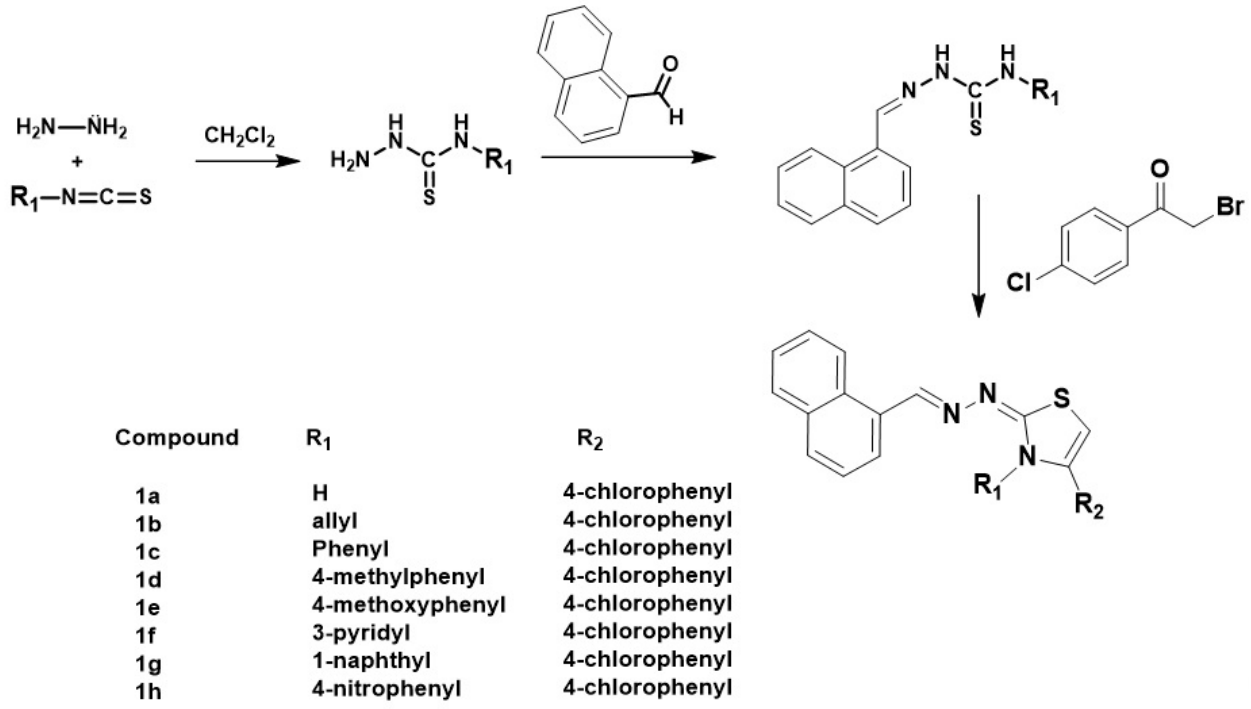
MATERIALS AND METHODS

4-(4-chlorophenyl)thiazole compounds

The compounds were synthesized at the Laboratory of Chemistry and Therapeutics Innovation (LQIT) at the Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil. The synthesis and chemical characterization were recently published by Cruz Filho et al. (2023). Briefly, the compounds were obtained in three steps: obtaining thiosemicarbazides synthesized from hydrazine hydrate and substituted isothiocyanate in dichloromethane (Step I). Thiosemicarbazones were obtained from the reaction of thiosemicarbazides with 1-naphthyl-carboxaldehyde (Step II). Finally (Step III), thiazoles (1a -h) were obtained from thiosemicarbazones, which were submitted to Hantzsch condensation with 2-bromo-4'-chloroacetophenone. Figure 1 presents the synthesis diagram for the compounds.

In silico evaluation of absorption, distribution, metabolism, excretion and toxicity parameters (ADMET)

The prediction of *in silico* ADMET parameters was performed according to the methodology proposed by Pires et al. (2015) and Daina et al. (2017). Initially, the SMILES codes (Simplified Molecular Input Line Entry Specification) were obtained through the free Swissadme platform (http://www.swissadme.ch/index.php) for each of the evaluated compounds. After obtaining the codes, these were entered into the also free pkCSM platform (http://biosig.unimelb.edu.au/



Compound	R_1	R_2
1a	H	4-chlorophenyl
1b	allyl	4-chlorophenyl
1c	Phenyl	4-chlorophenyl
1d	4-methylphenyl	4-chlorophenyl
1e	4-methoxyphenyl	4-chlorophenyl
1f	3-pyridyl	4-chlorophenyl
1g	1-naphthyl	4-chlorophenyl
1h	4-nitrophenyl	4-chlorophenyl

Figure 1. Synthesis diagram of 4-(4-chlorophenyl)thiazole 1a-1h.

pkcsm). Finally, all parameters obtained by the platform were evaluated.

Cell viability and proliferation assays

The experiments were carried out according to the methodology proposed by Qi et al. (2020) and Araújo et al. (2022) with modifications. Splenocyte cells (10^6/mL) were incubated for 24 hours with different concentrations of compounds (10 to 0.625 µM). Treated and untreated cells were then centrifuged at 450xG at 4 °C for 10 min. After discarding the supernatant, 1 mL of 1X PBS was added to the precipitate and after resuspension, the cells were centrifuged again (450 xG, 4°C, 10 min). The pellet was resuspended in 300 µL of binding buffer (10 µM HEPES at pH 7.4, 150 µM NaCl, 5 µM KCl, 1 µM $MgCl_2$ and 1.8 µM $CaCl_2$), transferred to a labeled cytometer tube and annexin V conjugated with fluorescein isothiocyanate (FITC) (1: 500) and propidium iodide (PI, 20 µg/mL). For determination of proliferation, cells were labeled with CFSE at the same concentrations as cell viability. The experiments were carried out in a FACSCANTO II flow cytometer, BDBiosciences.

In addition to cell viability and proliferation, the concentration of nitric oxide produced during cultivation by the Griess colorimetric method was quantified through culture supernatants of cells incubated or not. The experiment was carried out using 100 µL of culture supernatants and 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine-diHCl in 2.5% H_3PO_4). After 10 min at 25 ºC the absorbance was determined at 595 nm in an ELISA microreader. The NO dosage standard was performed using $NaNO_2$ (3.12-100 µM). This study was approved by the Committee on Ethics in the Use of Animals of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz, protocol number 164/2020.

In vitro anti-*P. falciparum* activity

The experiments were performed using the chloroquine-sensitive *P. falciparum* 3D7 strain,

according to the methodology proposed by Santos et al. (2023) with few modifications. Parasites were cultured at 5% hematocrit, 37 °C and 5% CO_2 atmosphere, human serum was replaced by 0.5% AlbuMAXII (Invitrogen ™) in the culture medium. Synchronized cultures were obtained by consecutive treatments at 48h intervals with 5% (w/v) D-sorbitol solution.

All compounds were evaluated for their *in vitro* antimalarial activity against *P. falciparum* strain 3D7. An unsynchronized culture with 1% hematocrit and 0.6% parasitemia was incubated (37 °C and 5% CO_2) with the tested compounds in concentrations ranging from 10 to 0.014 µM for 72 h. Compounds were dissolved in 1% DMSO. Parasite growth was assessed by flow cytometry (Beckman Coulter, Cytoflex) with a 96-well plate reader, using Fl-1 (green fluorescent protein [GFP]; excitation wavelength, 488 nm). Typically, 20,000 to 40,000 RBCs were counted for each well.

The effective concentration that promotes 50% inhibition of parasite growth (IC_{50}) was determined by non-linear regression using GraphPad Prism 5 software (trial version).

Statistical analysis

Data were analyzed using Sigma software, version 2.23.03. The normality of quantitative variables was performed using the Kolmogorov-Smirnov test. To detect differences between groups, the Wilcoxon test was used. The t-Student test was used to analyze the results of the cell viability assay. All results were expressed as mean (standard deviation) and a *p*-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In silico prediction of pharmacokinetic and toxicological parameters (ADMET)

The *in silico* prediction of ADMET parameters allows an assessment of the expected pharmacokinetic and toxicological profile for candidate compounds for drug prototypes (Pires et al. 2015). It is an alternative and easy-to-perform method that helps in the selection of compounds that have better pharmacotherapeutic profiles (Daina et al. 2017). Table I presents the prediction results for the parameters of absorption, distribution, metabolism, excretion and toxicity (ADMET), predicted by the free pkCSM platform.

The prediction results presented in Table I for the absorption parameters the first characteristic evaluated was the solubility in water (LogS). For a drug to be absorbed and pass into the bloodstream, it must be dissolved in biological fluids (Pires et al. 2015, Dascălu et al. 2020). The results show that the compounds were classified as sparingly soluble (−10 < sparingly soluble < −6), whereas chloroquine was classified as moderately soluble (−6 < moderate < −4). Permeability in Caco-2 cells is a widely used method to assess the intestinal absorption of orally administered drugs (Cabrera-Pérez et al. 2018). The compounds (1a to 1g) and chloroquine showed a moderate permeability profile. The compound 1h showed low permeability. In addition, all evaluated compounds showed a high probability of absorption in the human small intestine, with values > 80%. Regarding skin permeability, all compounds showed high permeability (logKp < −2.5).

Finally, the compounds were evaluated as substrates and inhibitors of p-glycoprotein and its isoforms. These glycoproteins work as an efflux pump against xenobiotics, protecting the body from the action of certain drugs (Yalcin 2020). The function of the glycoprotein is to prevent the entry of drugs into the cell or promote their elimination, depending on their location (Pires et al. 2015, Yalcin 2020). All compounds and chloroquine were classified as substrates. Only compound 1g and

Table I. Absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters predicted by the free pkCSM platform.

ADMET properties	1a	1b	1c	1d	1e	1f	1g	1h	Unit
Absorption									
Water solubility	-6.14	-6.66	-7.62	-7.60	-7.38	-6.35	-7.16	-7.11	Numeric (log mol/L)
Caco2 permeability	1.05	1.11	1.09	1.03	1.05	1.14	1.04	0.28	Numeric (log Papp in 10^{-6} cm/s)
Intestinal absorption	92.09	95.17	95.91	95.56	95.92	98.17	96.24	94.70	Numeric (%Absorbed)
Skin Permeability	-2.72	-2.67	-2.73	-2.73	-2.73	-2.72	-2.73	-2.73	Numeric (log Kp)
P-glycoprotein substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
P-glycoprotein I inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Categorical (Yes/No)
P-glycoprotein II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
Distribution									
VDssa	0.22	0.60	0.17	0.15	0.09	0.77	-0.30	-0.01	Numeric (log L/kg)
Fraction unbound	0.04	0.11	0.24	0.26	0.27	0.24	0.30	0.25	Numeric (Fu)
BBB permeability	0.52	0.28	0.42	0.50	0.48	0.71	0.59	-0.89	Numeric (log BB)
CNS permeability	-0.93	-1.02	-0.64	-0.61	-0.72	-1.07	-0.41	-1.06	Numeric (log PS)
Metabolism									
CYP2D6 substrate	No	No	No	No	No	No	No	No	Categorical (Yes/No)
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
CYP1A2 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Categorical (Yes/No)
CYP2C19 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Categorical (Yes/No)
CYP2C9 inhibitor	Yes	Yes	No	No	Yes	Yes	No	Yes	Categorical (Yes/No)
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	Categorical (Yes/No)
CYP3A4 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Categorical (Yes/No)
Excretion									
Total clearance	0.20	0.25	0.12	0.072	0.16	0.27	0.28	0.15	Numeric (log mL/min/kg)
Renal OCT2 substrate	No	No	No	No	No	No	No	No	Categorical (Yes/No)
Toxicity									
AMES toxicity	Yes	Yes	No	No	No	No	No	No	Categorical (Yes/No)
Maximum tolerated dose	0.28	0.53	0.61	0.60	0.63	0.43	0.49	0.50	Numeric (log mg/kg/day)
hERG I inhibitor	No	No	No	No	No	No	No	No	Categorical (Yes/No)
hERG II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
Oral rat acutee Toxicity	2.11	1.81	2.11	2.13	2.34	2.18	2.64	2.67	Numeric (mol/kg)
Oral rat chronicf Toxicity	1.06	0.85	0.35	0.34	0.71	0.69	-0.06	0.69	Numeric (log mg/kg_bw/day)
Hepatotoxicity	Yes	No	No	No	Yes	No	No	No	Categorical (Yes/No)
Skin Sensitization	No	No	No	No	No	No	No	No	Categorical (Yes/No)
T. Pyriformis toxicity	0.51	0.44	0.28	0.28	0.28	0.28	0.28	0.28	Numeric (log µg/L)
Minnow toxicity	-1.47	-3.74	-3.99	-3.73	-4.93	-1.47	-5.16	-3.84	Numeric (log mM)

Papp: Apparent permeability; BBB: Blood-Brain Barrier; CYP: Cytochrome P450; OCT2: Organic cation transporter 2; hERG: Human Ether-a-go-go Related Gene.

chloroquine were classified as non-inhibitors of P-glycoprotein I (it is a drug transporter). Regarding P-glycoprotein II (it is related to the biliary efflux of phosphatidylcholine) only chloroquine was classified as non-inhibitor.

The second parameter to be predicted was distribution. The volume of distribution (VDss) indicates the theoretical volume that a total dose would need to be uniformly distributed in plasma at the same concentration as observed in blood plasma. A high volume of distribution is expected when VDss (log VDss) > 0.45, indicating good distribution from plasma to tissues, while VDss values < -0.15 suggest drug distribution in plasma (Pires et al. 2015). Compounds (1a to 1f) and chloroquine are likely to be more easily distributed in tissues, whereas compounds 1g and 1h tend to be distributed in plasma. The

compounds showed low generation not bound to serum proteins.

Regarding permeability in the blood-brain barrier (BB). Compounds with logBB values > 0.3 easily cross the blood-brain barrier while compounds with logBB < −1 are poorly distributed to the brain (Pires et al. 2015). Only the 1h compound can be maldistributed in the BB. Regarding permeability in the central nervous system (CNS). Compounds with logPS values > -2 are considered CNS, while those with logPS < -3 are considered incapable of penetrating the CNS (Pires et al. 2015). All compounds and chloroquine were considered to be penetrants.

The third parameter evaluated was metabolism. The compounds were classified as inhibitors and substrates of cytochrome P450 and its isoforms. These proteins are heme-proteins involved in the biotransformations of various compounds of endogenous and exogenous origin (Wu et al. 2019). Biologically, these enzymes promote the chemical modification of several exogenous lipophilic molecules, which after that become more soluble and easily excreted by the human body (Pires et al. 2015, Wu et al. 2019). Only chloroquine has been classified as a CYP2D6 substrate. All compounds and chloroquine were considered CYP3A4 substrates. Only the compounds 1g 1h and chloroquine are not CYP1A2 inhibitors. Compounds 1g and chloroquine were considered as non-CYP2C19 inhibitors. Regarding the CYP2C9 isoform, compounds 1c, 1d 1g and chloroquine were considered as non-inhibitors. For the CYP2D6 isoform, only chloroquine was considered as an inhibitor. Finally, for the CYP3A4 isoform, only 1g, 1h and chloroquine were considered non-inhibitors.

Regarding excretion, compounds and chloroquine have low clearance values. Furthermore, only chloroquine was considered as a Renal OCT2 substrate.

Finally, the last pattern evaluated was toxicity. Only compounds 1a, 1b and chloroquine were positive for the AMES test, indicating a possible mutagenic effect. For a given compound, an MRTD less than or equal to 0.477 log(mg/kg/day) is considered low and high if greater than 0.477 log(mg/kg/day). Only compound 1a and chloroquine are considered low. All compounds were non-hERG I inhibitors and hERG II inhibitors. Regarding acute toxicity, the compounds and chloroquine showed values ranging from 1.81 to 2.85 mol/kg. Chronic toxicity ranging from -0.06 to 1.06 log mg/kg_bw/day. Only compounds 1a, 1e and chloroquine were considered hepatotoxic. The compounds are not sensitive to the skin. Toxicity in relation to *T. Pyriformis* the compounds and chloroquine promoted toxicity ranging from 0.28 to 1.55 log µg/L. Negative log results of the concentration required to inhibit 50% growth in log µg/L) is predicted, with a value > -0.5 log µg/L considered toxic. Therefore, all compounds and chloroquine were considered toxic. Regarding Minnow, the compounds promoted toxicity ranging from -5.16 to 0.74 mM. Log LC_{50} values < -0.3 are considered to be of high acute toxicity. Only chloroquine was considered toxic.

Through the *in silico* analysis, it was possible to verify that the compounds can present a promising kinetic drug profile and can be considered possible drug candidates. However, it is worth noting that the study carried out here is *in silico* and *in vitro* and *in vivo* tests need to be performed to propose these compounds as drugs.

Cell viability and proliferation assays

Cytotoxicity results were obtained through assays using annexin V (apoptosis) and propidium iodide (necrosis). Annexin V binds by affinity to the membrane phospholipid phosphatidylserine which is normally found on the inner side of the

membrane (Crowley et al. 2016). When the cell is in early apoptosis, the membrane phospholipid phosphatidylserine is translocated to the outside, thus binding Annexin V (Moore et al. 1998). As for the assay using propidium iodide, it is known that this compound is able to penetrate the cell when there is a change in membrane permeability and, once inside the cell, binds to DNA, producing fluorescence (Crowley et al. 2016). Membrane permeability alteration is a characteristic of cell necrosis and also of late apoptosis (Moore et al. 1998). The fluorescence emission, for both assays, can be analyzed using the cytometry technique.

The curves shown for the compounds and chloroquine are shown in Figures S1, S2, S3 and S4 of the supplemental material respectively. The results showed that the compounds and chloroquine were not capable of promoting cytotoxic effects against splenocytes at the evaluated concentrations (viability > 95%). These results show that the compounds evaluated here are not cytotoxic against splenocyte cells under the conditions evaluated here. These compounds were previously studied by Cruz Filho et al. (2023) evaluating different cell types. The authors obtained IC_{50} results ranging from 45.12 to values greater than 200 µM for macrophage cells, 68.9 to greater than 200 µM against fibroblasts, 73.4 to greater than 200 µM for HepG2 cells and hemolysis values lower than 10% (non-hemolytic) indicating that these compounds are of low toxicity.

In parallel, cytotoxicity was determined by the production of nitric oxide promoted by cells treated with compounds and chloroquine, using the Griess colorimetric method. This method is based on the reaction between nitrite and an aromatic amine in acidic solution to form an intermediate diazonium salt (diazotization). Next, the coupling of the diazonium ion with an aromatic compound that has an amino substituent group or a hydroxyl group occurs, forming the colored azo compound (Yucel et al. 2012). The results of the determination of nitric oxide produced by cells treated with the compounds and chloroquine showed that these were able to promote an increase in nitric oxide without promoting cell death (Figure S3 and Figure S4 of the supplemental material respectively). This fact may be related to the activation of immune cells and a possible mechanism of the anti-inflammatory response (Granger & Kubes 1996).

Finally, the CFSE assay showed an increase in the proliferation of cells treated both with the compounds (Figure S5 of the supplementary material) and with chloroquine (Figure S6). This assay is based on CFSE binding to cells and at each division, cell fluorescence drops by half, which makes this dye an ideal tool to monitor cell proliferation. Each daughter cell inherits approximately half of the CFSE labeling, thus allowing the tracking and quantification of cell divisions (Banks et al. 2011). Therefore, the activation and proliferation of immune cells is an important factor for combating infections promoted by different parasites.

Anti-*P. falciparum* activity *in vitro*

One of the characteristics of compounds with antiparasitic activity is toxicity. Drug candidate compounds should show less toxicity to animal cells when compared to different parasites (Santos et al. 2023). All compounds evaluated in our study showed low cytotoxicity against mammalian cells. Based on this result, these compounds were tested against the chloroquine-sensitive parasite *P. falciparum* 3D7. Table II presents the IC_{50} results for each of the compounds compared to chloroquine.

The results presented in Table II show that the compounds except compound 1f were active against the parasite *P. falciparum*. Regarding IC_{50}

Table II. Results of *in vitro* anti-*P. falciparum* activity expressed in IC_{50} for each of the compounds compared to chloroquine.

Compounds	IC_{50} (μM) Mean (Standard deviation)
1a	3.62(0.0)
1b	3.81(0.2)
1c	2.23(0.01)
1d	1.24(0.01)
1e	1.62(0.02)
1f	>10
1g	2.20(0.03)
1h	0.79(0.01)
Chloroquine	0.75(0.0)

values, the compounds showed values ranging from 0.79 to greater than 10 μM, respectively. The low toxicity against mammalian cells and high toxicity against the parasite indicates that these compounds are more selective against the parasite when compared to mammalian cells. In addition, all compounds showed lower values than chloroquine. However, they showed activity at non-toxic concentrations to mammalian cells, proving to be promising antiparasitic agents.

The literature presents different results promoted by compounds against the parasite *P. falciparum*, these different values are associated with the chemical structure of these compounds. Makam et al. (2014) evaluating 2-(2-hydrazinyl) thiazole compounds. They obtained IC_{50} results ranging from 0.648 to greater than 10 μM. Cohen et al. (2012) obtained values ranging from 4.8 to greater than 25 μM for aphtho [2, 1-d] thiazole compounds. Santos et al. (2023) found that thiazole compounds were able to promote growth inhibition with IC_{50} results ranging from 0.47 to 4.11 μM, except for compounds 2g, 2l and 2p that did not promote growth inhibition. These findings show that the compounds evaluated here are promising antimalarial agents.

CONCLUSION

The results presented in this work allowed concluding that the compounds 4-(4-chlorophenyl)thiazole, have favorable pharmacokinetic properties, are of low toxicity, under the evaluated experimental conditions, were able to activate (increase of nitric oxide without promoting cell death) and proliferate splenic cells. Furthermore, they were able to inhibit the growth of the *P. falciparum* parasite. These findings show that these compounds are potential candidates for antimalarials.

Competing interest statement

The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this article.

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SUPPLEMENTARY MATERIAL

Figures S1 – S6.

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