Malaria Journal

RESEARCH

Open Access

Unravelling the mode of action of the Tres Cantos Antimalarial Set (TCAMS): investigating the mechanism of potent antimalarial compounds potentially targeting the human serotonin receptor

Benedito Matheus dos Santos[1], Lenna Rosanie Cordero Mallaupoma[1,2], Mateus Fila Pecenin[1], Abhinab Mohanty[1], Angela Lu[3], Paula J. Bartlett[3], Andrew P. Thomas[3], Francisco-Javier Gamo[4] and Celia R. S. Garcia[1*]

Abstract

Background Despite the strides made in recent decades, the resistance observed in existing antimalarial drugs, and the intricate life cycle of the *Plasmodium* parasite underscore the pressing need to develop novel and effective therapeutic interventions. This article provides a comprehensive evaluation of the outcomes stemming from screening a library comprising 48 compounds (TCAMS) against *Plasmodium falciparum*.

Methods This study focused on characterizing the IC_{50} values of compounds from the Tres Cantos Antimalarial Set (TCAMS) library via a double-labelling method of *P. falciparum* parasites with SYBR Green-I and MitoTracker Deep Red, which were evaluated via flow cytometry. Evaluation of the cytotoxicity of the best candidates in human embryonic kidney (HEK293) cells, chemoinformatic analysis, and exploration of the effects of the compounds on the action of serotonin and melatonin in the erythrocytic life cycle of the parasite.

Results IC_{50} characterization confirmed that 93.75% of the compounds tested exhibited antimalarial activity at concentrations below 2 micromolar (µM), with 5 compounds showing IC_{50} values below 50 nM (nM) (15.21 ± 5.97 nM to 45.82 ± 5.11 nM). Furthermore, 12 compounds presented IC_{50} values between 50 and 100 nM (57.43 ± 12.25 nM to 100.6 ± 22.89 nM), highlighting their potent in vitro efficacy against *P. falciparum*. Cytotoxicity evaluation in HEK293 cells revealed that 12 from 17 compounds did not significantly reduce cell viability. Cheminformatics analysis clustered the compounds based on structural and physicochemical similarities, revealing distinct structural patterns. Exploration of hypothetical targets from the TCAMS library identified 27 compounds with potential targets, 15 specifically targeted serotonergic receptors. Subsequent serotonin and melatonin treatment experiments indicated that certain compounds could inhibit both effects on parasitaemia, suggesting a complex interaction with signaling in *P. falciparum*.

*Correspondence:
Celia R. S. Garcia
cgarcia@usp.br
Full list of author information is available at the end of the article

Conclusions This study identifies promising antimalarial candidates with low IC_{50} values and highlights the significance of targeting serotonin receptors in the development of potential antimalarial drugs.

Keywords Antimalarial, Melatonin, *Plasmodium falciparum*, Serotonin, TCAMS

Background

Apicomplexa parasites belonging to the *Plasmodium* genus are the causative agents of malaria, a globally significant and widespread infectious disease [1–3]. As of 2023, the World Health Organization (WHO) estimated that there were approximately 263 million malaria cases, resulting in approximately 597,000 deaths across 85 endemic countries worldwide. The majority of these cases are concentrated in Africa, where approximately 94% of malaria-related deaths occur [4]. In 2023, the number of reported cases rose to 247 million, surpassing pre-COVID-19 pandemic estimates and indicating a slight increase compared with previous years [4, 5].

Despite the effectiveness of standard-of-care (SoC) antimalarial drugs in treating and preventing malaria, the emergence of drug resistance poses a growing challenge in affected regions, particularly in sub-Saharan Africa and Southeast Asia [6, 7]. Drug resistance develops when the *Plasmodium* parasite acquires the ability to survive, reproduce, and be transmitted even in the presence of therapeutic drug doses [8–11]. The limited diversity of available antimalarial drugs is one contributing factor (in fact, artemisinin-based combination therapy is the only SoCs recommended in most endemic countries), as the excessive and repetitive use of the same drugs can lead to natural selection of resistance [11, 12].

Addressing the need for new antimalarial treatments requires a collaborative effort that integrates the expertise of professionals, researchers, academic institutions, and the pharmaceutical industry [13–15]. In this context, the introduction of the Tres Cantos Antimalarial Set (TCAMS) presents a diverse array of chemical compounds derived from the compound bank of the pharmaceutical company GlaxoSmithKline (GSK), which is dedicated to the exploration and discovery of novel antimalarial drugs [16].

The TCAMS library originated from an initial study screening of 1,986,056 compounds from the GSK screening collection. Among these compounds, 13,533 exhibited in vitro antiplasmodial activity, demonstrating the ability to inhibit the erythrocytic development of the *Plasmodium falciparum* 3D7 strain by at least 80% at a concentration of 2 µM. Additionally, over 8,000 compounds displayed potent activity against the multidrug-resistant strain Dd2 of *P. falciparum* [16].

Through collaborations between GSK and academic institutions [17]. A collective effort has led to the exploration of numerous promising chemotypes for potential antimalarial candidates, including cyclopropyl carboxamides [18, 19], spiroindolones [20], indolines [21], aminohydantoins [22], 2-phenoxyanilides [23], carbamoyl triazoles [24] and other unexplored classes that hold potential for the development of innovative antimalarial therapies.

Employing a subset of 48 compounds from the TCAMS library, the survival and erythrocytic proliferation of *P. falciparum* 3D7 via a dual-labelling flow cytometry technique were assessed [25]. This technique involves I: SYBR Green-I for DNA labelling and II: MitoTracker Deep Red as a mitochondrial marker. Given the membrane potential dependence of the latter, measures were taken to label and select viable parasites exclusively. The IC_{50} values for each compound were determined, and their cytotoxic activity in HEK293 cell cultures was investigated.

Through cheminformatics analysis, compounds based on structural similarities pinpointed hypothetical targets for 27 out of the 48 compounds under scrutiny were categorized. Specifically, 15 compounds that act as serotonin receptor agonists or antagonists were identified. These compounds were then selected to examine their impact on the antimalarial mechanism of action of the neurotransmitter serotonin and the host hormone melatonin within the erythrocytic cycle of *P. falciparum*. A detailed analysis of one serotonergic TCAMS compound with respect to erythrocytic cycle stage specificity and comparison to chloroquine were made.

Methods

Cultivation and maintenance of *P. falciparum*

Red blood cells infected with *P. falciparum* parasite strain 3D7 and a serpentine receptor 25 knockout strain ($PfSR25^{-}$) were grown in 175 cm^2 culture flasks (Greiner Bio-One) containing RPMI 1640 (Gibco—Thermo Fisher) supplemented with 0.5% NaHCO3, 0.04% gentamicin sulfate, 0.05% hypoxanthine and 0.5% AlbuMAX I [26]. The culture flasks were maintained at 37 °C in an atmosphere of 90% N2, 5% O_2, and 5% CO_2. Parasitaemia was determined and monitored by blood smears stained with Rapid Panoth (Laborclin).

Incubation tests of the compounds in *P. falciparum* cultures

The TCAMS-GSK compound library (48 compounds) was diluted in cell culture grade DMSO (Sigma–Aldrich)

at a stock concentration of 2 mM and stored at −20 °C until use. To perform the experiments and determine the IC_{50} values for each compound, different final concentrations were used (0.0019 µM, 0.0039 µM, 0.0078 µM, 0.0156 µM, 0.0312 µM, 0.0625 µM, 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, and 2 µM). The compounds were incubated with asynchronous cultures of *P. falciparum* with 0.3% initial parasitaemia and 2% haematocrit for 72 h. DMSO (0.13% v/v) was used as a solvent control, and the antimalarial drug chloroquine (0.2441–250 nM) was used as a positive control for growth inhibition.

Parasite labelling and flow cytometry assay

Parasitaemia of *P. falciparum* cultures was quantified by flow cytometry as described in EKLAND; SCHNEIDER; FIDOCK, 2011 [25]. After 72 h of incubation, the parasites were labelled with the nucleic acid marker SYBR Green-I (1: 10,000) and the membrane potential marker MitoTracker Deep Red (50 nM), both of which were diluted in phosphate-buffered saline (PBS). After 20 min of incubation at 37 °C, the final parasitaemia was obtained by reading the plates via a BD Accuri C6 cytometer (Becton Dickinson).

The results were converted into dot plots and analysed via FlowJo-V10.9 software (FL4-H through FL1-H). All the assays were performed in triplicate (n = 3 experiments), and the percentage of parasite survival was calculated relative to the DMSO solvent (v/v). GraphPad Prism software was used to calculated the dose–response curves and mean 50% inhibitory concentration (IC_{50}) values. The IC_{50} values for each compound were calculated in µM following the calculations used to construct the dose–response survival curves. They were finally converted from µM to nM to facilitate interpretation of the results.

Cultivation and maintenance of HEK293 cells

Human embryonic kidney cells (HEK293) were maintained in 75 cm^2 culture flasks (Greiner Bio-One) at 37 °C with 5% CO_2 containing Dulbecco's minimal essential medium (DMEM) plus 10% fetal bovine serum, 1% streptomycin and penicillin (Gibco—Thermo Fisher Scientific), and 3.7 g/l $NaHCO_3$ (Sigma). Cells from passages 7–12 harvested with trypsin–EDTA were used in the experiments. The number of cells was monitored by counting in a Neubauer chamber coupled with an inverted microscope (Zeiss).

Evaluation of the cytotoxicity of the compounds in HEK293 cells

To assess the cytotoxicity of the compounds, HEK293 cells were trypsinized and plated in 96-well flat-bottom plates at a density of 10^4 (10,000) cells per well and incubated for 24 h at 37 °C and 5% CO_2. After plating, the cells were incubated with a range of concentrations of the test compounds (0.0019–2 µM).

After 72 h of incubation at 37 °C and 5% CO_2, 40 µL of MTT reagent (thiazolyl blue tetrazolium bromide, 5 mg/mL) was added to each well, and the cells were incubated for an additional 3 h at 37 °C and 5% CO_2. The medium was then removed, 100 µL of DMSO/well was added, and the plates were homogenized on a shaker for 15 min. The absorbance of each well was quantified at a wavelength of 570 nm via a FlexStation 3 instrument (Molecular Devices). Survival (dose–response) curves were obtained by analysing the data via GraphPad Prism software. Cells incubated with DMSO (v/v) were used as negative controls (noncytotoxic). All the assays were performed in triplicate and were repeated in 3 independent experiments.

The ChemMine tools platform was used to analyse the TCAMS library

ChemMine Tools is an online service for the analysis of small-molecule data. It provides a web interface for cheminformatics tools [27]. The ChemMine platform was used to perform the cheminformatics analyses. First, the compound library was uploaded to the platform by entering each compound's names and SMILES sequences. Once the compound library was loaded, the tools provided by ChemMine were used to perform various analyses. The physicochemical property tool JoeLib was chosen to process the compounds. This tool calculates 38 physicochemical descriptors for each compound using the JoeLib library.

The generated data were then used to group the compounds. The platform was subsequently used to perform multidimensional scaling (MDS) clustering with the following parameters: similarity cutoff: 0.5, graph dimensions: 3D. The distance matrices required for MDS clustering are computed as all-against-all comparisons via atom pair similarity measures, and the generated similarity scores are transformed into distance values. The scatter plot was generated via the CanvasXpress JavaScript library.

Effects of TCAMS library compounds hypothetically targeting serotonin receptors on the erythrocytic cycle of *P. falciparum*

Erythrocytes infected with *P. falciparum* 3D7 1% initial parasitaemia and 2% haematocrit were incubated with 2.5 nM of the serotonergic compounds present in the TCAMS-GSK library (agonists: TCMDC-139803, TCMDC-140136, TCMDC-139482, TCMDC-139634, TCMDC-139786, TCMDC-139813, TCMDC-140219 and antagonists: TCMDC-139860, TCMDC-142311,

TCMDC-142310, TCMDC-135301, TCMDC-135459, TCMDC-135267, TCMDC-135435, TCMDC-135385 of the serotonin receptor), 100 nM melatonin and 250 nM serotonin (Sigma) for 48 h at 37 °C under mixed gases. The control group was incubated with DMSO (0.025%). To investigate the effects of serotonergic compounds on the action of the hormone and neurotransmitter serotonin, the compounds were incubated together with 100 nM melatonin and 250 nM serotonin under the same conditions as mentioned above. After 48 h of incubation, parasitaemia was determined as described in Sect. 2.3 Parasite labelling and flow cytometry assay. All the assays were performed in triplicate (3 independent experiments), and the percentage of parasite survival was calculated in relation to the solvent (v/v).

Evaluation of drug effects on erythrocytic cycle phases

Cultures of *Plasmodium falciparum* 3D7 were synchronized using sorbitol (PMID: 383,936) and seeded into 96-well plates at 1% parasitaemia and 2% haematocrit. Drugs or vehicle control (DMSO) were added at the indicated time points, and the cultures were incubated for 48 h. Samples were collected from the incubations after 12, 24, 36, and 48 h post-synchronization. These samples were washed and incubated for 30 min in HEPES-buffered Hank's solution (in mM: 25 HEPES, 121 NaCl, 5 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 10 glucose) containing 1 µM Hoechst and 400 µM thiazole orange to stain DNA and RNA, respectively (PMID: 18,302,186). Parasitaemia and the distribution of rings, trophozoites, and schizonts were determined using flow cytometry (BD LSRFortessa X-20) with gating based on RNA and DNA staining levels.

Results

Characterization of the IC_{50} values and evaluation of the cytotoxicity of the compounds in the TCAMS library

The IC_{50} values for each compound were determined in asynchronous cultures of *P. falciparum* 3D7 exposed to various concentrations of the test compounds (0.0019–2 µM). Parasitaemia was assessed by flow cytometry via a double-labelling technique: SYBR Green-labelled DNA and MitoTracker Deep Red-labelled mitochondria. The latter dye is membrane potential-dependent, ensuring that only viable parasites are labelled. Table 1 shows the SMILES formatted structures and IC_{50} values for the 48 compounds tested and the chloroquine control.

Results of the screening of compounds from the TCAMS library against *P. falciparum* 3D7

Molecular structure of the compounds in SMILES format and IC_{50} values ± standard error of the.

Among the 48 compounds evaluated, 93.75% (45) showed in vitro antimalarial activity against 3D7 at concentrations less than 2 µM. The compounds TCMDC-135334, TCMDC-139894, and TCMDC-140381 could not reduce *P. falciparum* parasitaemia by 50% at the concentrations tested (0.0019–2 µM). Analysis of the IC_{50} values obtained after screening revealed five compounds with IC_{50} values very close to and/or even lower than those of antimalarial drugs used for the clinical treatment of malaria [28]. Figure 1 shows the survival curves and IC_{50} values for the best hits identified after screening (TCMDC-137476, TCMDC-142311, TCMDC-140136, TCMDC-138003, and TCMDC-139482) with IC_{50} values ≤ 50 nM.

Overall, this screening identified five compounds with IC_{50} values below 50 nM and characterized 12 compounds with IC_{50} values between 50 and 100 nM, ranging from 57.43 ± 12.25 nM (TCMDC-139867) to 100.6 ± 22.89 nM (TCMDC-140219). Twenty-one compounds presented IC_{50} values ranging from 109.80 ± 46.23 (TCMDC-136171) to 497.30 ± 99.52 (TCMDC-134778), and 7 compounds presented IC_{50} values greater than 500 nM: TCMDC-124574 (681.9 ± 213.10), TCMDC-135435 (743 ± 161.70), TCMDC-123874 (759.1 ± 130.70), TCMDC-131795 (909 ± 65.06), TCMDC-135385 (1210 ± 314.60), TCMDC-133589 (1359 ± 197.50) and TCMDC-132496 (1968 ± 115.50). The dose–response survival curves for all 45 compounds are shown in Supplementary Fig. 1.

After screening and IC_{50} characterization, the toxicity of 17 compounds with IC_{50} values up to 100 nM in mammalian HEK293 cells was evaluated. Viability and proliferation were assessed via the MTT colorimetric method. Dose–response survival curves for each compound are shown in Supplementary Fig. 2. The analysis revealed that most compounds (12) did not affect cell proliferation or viability, with viability values remaining above 90%, similar to those of the control group incubated with 0.13% DMSO (v/v). However, the compounds TCMDC-137476, TCMDC-140136, TCMDC-139867, TCMDC-138317, and TCMDC-137905 exhibited cytotoxic activity in HEK293 cells. The cytotoxic concentration values (CC_{50}) and selectivity calculations are shown in Table 2.

The results presented in Table 2 show that out of the five compounds investigated, four (TCMDC-137476, TCMDC-140136, TCMDC-139867, and TCMDC-137905) have a promising SI, as the values calculated for the SI were greater than 10. As noted by Ndjakou Lenta et al. [29], SI values above 10 indicate a more significant margin of safety, whereas values below 10 indicate toxicity. Consistent with these parameters, only TCMDC-138317 (SI = 0.20) was toxic to mammalian HEK293 cells, with a CC_{50} value of 16.61 nM, which is significantly

Table 1 Results of the screening of compounds from the TCAMS library against *P. falciparum* 3D7

Code TCMDC	SMILES	IC_{50} (nM) 3D7
TCMDC-137476	Cl.COc1ccc2nc3cc(Cl)ccc3c(NCCN3CCN(C)CC3)c2c1	**15.21** ± 5.97
TCMDC-142311	Fc1ccc(CN(CCCNc2c(NCc3cccnc3)c(=O)c2=O)c2ccccn2)cc1	**16.09** ± 5.87
TCMDC-140136	Cl.Clc1ccc(\C=C\C(=O)N2CCC(CCN3CCC(CC3)c3c[nH]c4cnccc34)CC2)cc1Cl	**23.56** ± 7.97
TCMDC-138003	OC(=O)C(O)=O.CCCN(CCCN(CCCN(CCC)C(C)C)c1cc(C)nc(Nc2ccc(Cl)cc2)n1)C(C)C	**26.58** ± 5.68
TCMDC-139482	Cc1[nH]c2ccccc2c1C1CCN(CC2CCC(CC2)NC(=O)\C=C\c2ccc(Cl)c(Cl)c2)CC1	**45.82** ± 5.11
TCMDC-139867	Cl.CC(Cc1ccc(NC(=O)c2ccc(CNCCc3ccccc3)cc2)cc1)NCCc1cccc(Cl)c1	**57.43** ± 12.25
TCMDC-139860	FC(F)(F)c1ccc2c(c[nH]c2c1)C1CCN(CC2CCN(CC2)C(=O)\C=C\c2ccc(Cl)c(Cl)c2)CC1	**65.08** ± 11.59
TCMDC-132918	OC(=O)C(F)(F)F.Fc1ccc2[nH]c(nc2c1)-c1cccc(c1)-c1ccc(CN2CCN(CC2)c2ccncc2)cc1	**68.43** ± 6.93
TCMDC-134281	OC(=O)C(F)(F)F.Clc1ccc2nccc(N3CCC(CN4CCC(CC4)c4c[nH]c5ccccc45)CC3)c2c1	**72.89** ± 10.04
TCMDC-135346	COc1cc(NC(=O)N2CCOC(CCN3CCC4(CCc5ccccc45)CC3)(C2)c2ccc(Cl)c(Cl)c2)cc(OC)c1OC	**75.55** ± 18.21
TCMDC-125636	COc1ccc(cc1OC)C1CC(=O)C2=C(C1)NC(C)=C(C2c1cccc(O)c1)C(=O)OC(C)C	**80.24** ± 18.91
TCMDC-138317	Cl.CCOc1cc(CC)ccc1C1CCN(CCCCNC(=O)c2ccc(NC(=O)c3ccc(Cl)cc3)cc2)CC1	**80.79** ± 25.92
TCMDC-137905	Cl.CN(CCCNCc1cccc2ccccc12)CCCNCc1cccc2ccccc12	**91.13** ± 18.44
TCMDC-139803	Cc1[nH]c2c(C)cccc2c1C1CCN(CCC2CCN(CC2)C(=O)\C=C\c2ccc(Cl)c(Cl)c2)CC1	**91.86** ± 18.07
TCMDC-139502	COc1cc(C)nc2ccc(NC(=O)c3ccc(cc3)C(=O)Nc3ccc4nc(C)cc(OC)c4c3)cc12	**98.15** ± 25.08
TCMDC-142310	Fc1ccc(CN(CCCNc2c(NCc3ccncc3)c(=O)c2=O)c2ccccn2)cc1	**100** ± 23.64
TCMDC-140219	Cc1[nH]c2ccc(O)cc2c1C1CCN(CC2CCN(CC2)C(=O)\C=C\c2ccc(Cl)c(Cl)c2)CC1	**100.6** ± 22.89
TCMDC-136171	Cc1cc(NC(=O)NCCN2CCC(CC2)NC(=O)c2ccc(Cl)cc2)c2ccccc2n1	**109.8** ± 46.23
TCMDC-139813	CS(=O)(=O)Nc1ccc2[nH]cc(C3CCN(CC4CCN(CC4)C(=O)\C=C\c4ccc(Cl)c(Cl)c4)CC3)c2c1	**116.1** ± 49.14
TCMDC-131917	Cl.COc1cc2nccc(Nc3ccc(Oc4ccccc4)cc3)c2cc1OC	**120.2** ± 19.39
TCMDC-139494	Oc1ccc2[nH]cc(C3CCN(CC4CCC(CC4)NC(=O)\C=C\c4ccc(Cl)c(Cl)c4)CC3)c2c1	**122.1** ± 21.14
TCMDC-139786	Oc1ccc2[nH]cc(C3CCN(CC4CCN(CC4)C(=O)\C=C\c4ccc(Cl)c(Cl)c4)CC3)c2c1	**138.3** ± 23.87
TCMDC-137541	Cl.CN1CCN(CCCCCNc2c3ccccc3nc3ccccc23)CC1	**165.2** ± 74.71
TCMDC-141946	CC[C@H](NC(=O)c1c(CC2CCN(CC(C)=O)CC2)c(nc2ccccc12)-c1ccccc1)c1ccccc1	**215.1** ± 27.89
TCMDC-139258	CCN(CCNc1nc(NCCc2cnc[nH]2)nc(n1)N(CCC#N)Cc1ccccc1)c1cccc(C)c1	**215.8** ± 15.41
TCMDC-132764	OC(=O)C(F)(F)F.FC(F)(F)c1ccc2[nH]c(nc2c1)-c1ccc(s1)-c1ccc(CNCCCN2CCCC2=O)cc1	**259.4** ± 49.40
TCMDC-139856	Clc1cccc(Cl)c1\C=C\C(=O)NC1CCC(CN2CCC(CC2)c2c[nH]c3ccccc23)CC1	**274.2** ± 31.92
TCMDC-133018	OC(=O)C(F)(F)F.Cn1nccc1C(=O)NCCNCc1ccc(cc1)-c1ccc(s1)-c1nc2cc(ccc2[nH]1)C(F)(F)F	**284.5** ± 125.2
TCMDC-140590	OC(=O)C(F)(F)F.O=C(Nc1ccccc1)N(Cc1cccc(c1)c1cccc(CNCc2ccc3OCOc3c2)c1)C1CCN(Cc2ccccc2)CC1	**284.7** ± 143.8
TCMDC-135459	COc1cc2nc(NCCc3ccccc3)nc(NCc3cnn(C)c3C)c2cc1OC	**287.1** ± 24.22
TCMDC-135829	Cl.COc1ccc2ncc(F)c([C@H](O)CN3CCC(CC3)NCc3ccc4OCC(=O)Nc4c3)c2c1	**335.6** ± 175.5
TCMDC-139634	Oc1ccc2[nH]cc(C3CCN(Cc4cccc(NC(=O)\C=C\c5ccc(Cl)c(Cl)c5)c4)CC3)c2c1	**345.7** ± 47.47
TCMDC-135267	COc1cccc(CCNc2nc(NCc3ccccc3)c3cc(OC)c(OC)cc3n2)c1	**389.4** ± 119.5
TCMDC-135301	COc1cc2nc(NCCc3ccccc3)nc(NCCCc3ccccc3)c2cc1OC	**433.6** ± 81.2
TCMDC-135338	COc1cc(NC(=O)N2CCOC(CCN3CCC4(CCc5ccccc45)CC3)(C2)c2ccc(Cl)c(Cl)c2)cc(OC)c1	**441.5** ± 78.85
TCMDC-141151	O=C1Nc2cccc3CCCC1(CCCCN1CCC(CC1)c1cc4ccncc4[nH]1)c23	**459** ± 33.75
TCMDC-135344	CCOc1ccc(cc1)C(=O)N1CCOC(CCN2CCC(CC2)c2ccccc2)(C1)c1ccc(Cl)c(Cl)c1	**462.5** ± 184.7
TCMDC-134778	OC(=O)C(F)(F)F.COc1ccc(NC(=O)[C@H](CC(C)C)Nc2cc(C)nc(NCCc3ccccc3)n2)cc1	**497.3** ± 99.52
TCMDC-124574	Brc1ccc(Nc2nc(NCc3ccccc3)c3ccccc3n2)cc1	**618.9** ± 213.1
TCMDC-135435	COc1cc2nc(NCCc3ccccc3)nc(NCc3cc(C)no3)c2cc1OC	**734** ± 161.7
TCMDC-123874	COc1ccc(CN2C(CC(=O)Nc3ccccc3)C(=O)N(Cc3ccccc3)C2=S)cc1OC	**759.1** ± 130.7
TCMDC-131795	CCCCCC(C)C(C)c1cc(O)c2c(OC(C)(C)CC2(O)c2ccnn2C)c1	**909** ± 65.06
TCMDC-135385	COc1cc2nc(NCCc3ccccc3)nc(N(C)Cc3ccccc3)c2cc1OC	**1210** ± 314.6
TCMDC-133589	Cc1ccc(NC(=O)Nc2ccc(Cl)c(c2)C(F)(F)F)cc1-c1ccc(cc1)C(=O)NCCN1CCOCC1	**1359** ± 197.5
TCMDC-132496	Cn1cnc(CCNC(=O)c2cccc(c2)-c2cccc(c2)-c2nc3cc(ccc3[nH]2)C(F)(F)F)c1	**1968** ± 115.5
TCMDC-139894	Oc1ccc(cc1C(=O)NC1CCC(CN2CCC(CC2)c2c[nH]c3ccccc23)CC1)-c1ccc(F)cc1F	–
TCMDC-135334	Fc1ccc(cc1)C(=O)N1CCOC(CCN2CCC(CC2)c2ccccc2)(C1)c1ccc(Cl)c(Cl)c1	–
TCMDC-140381	CC[C@H](NC(=O)c1c(CN2CCC(CC2)N2CCCCC2)c(nc2cc(O)ccc12)-c1ccccc1)c1ccccc1	–
Chloroquine	CCN(CC)CCCC(C)NC1=C2C=CC(=CC2=NC=C1)Cl	**11.33** ± **0.70**

Table 1 (continued)

Bold values represent the IC_{50}

Molecular structure of the compounds in SMILES format and IC_{50} values ± standard error of the mean

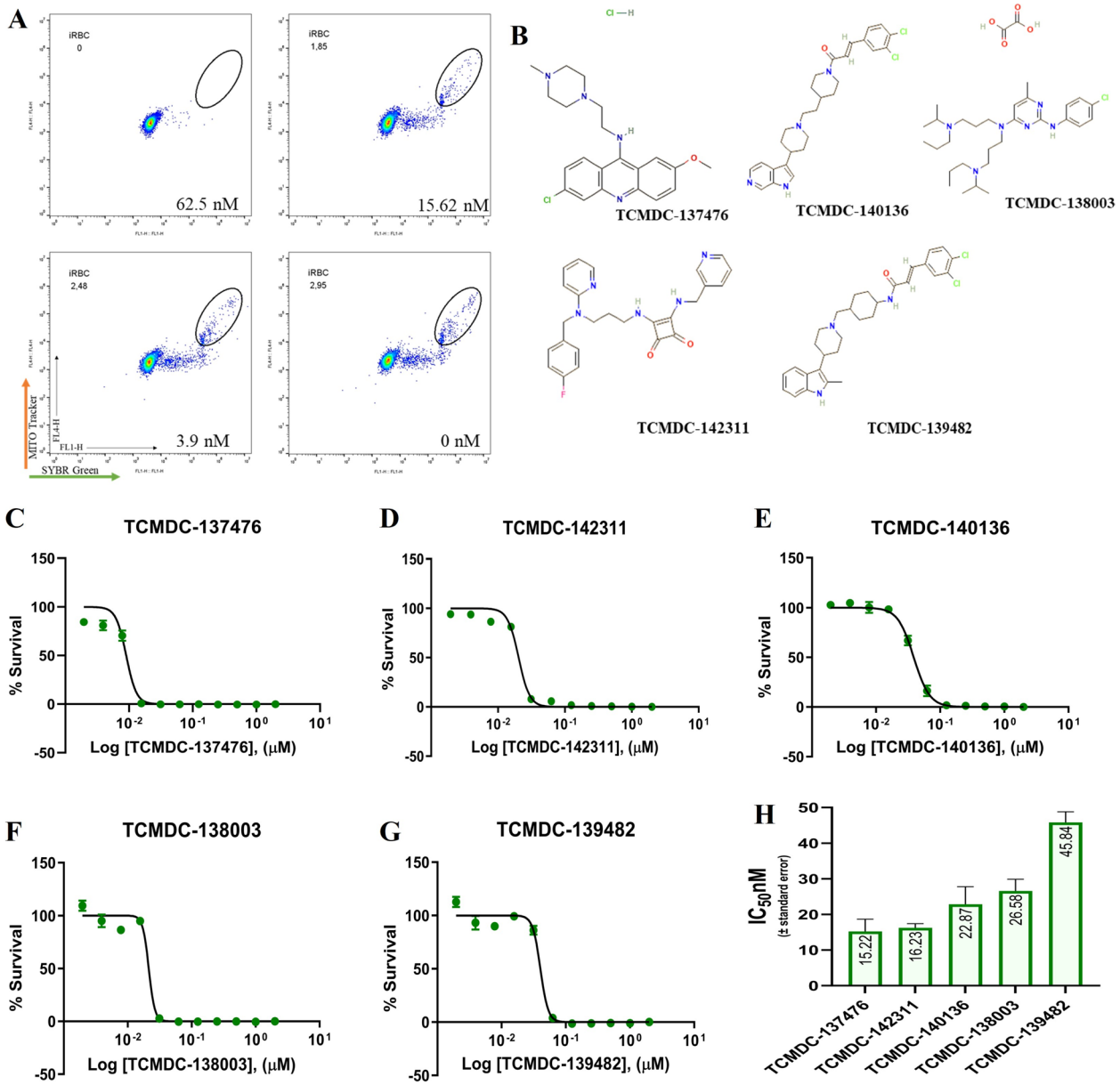


Fig. 1 Survival curves for the best hits identified in the screening of selected compounds from the TCAMS library. **A** Dot plot analysis: *P. falciparum* 3D7 parasites were stained with SYBR Green-I, which intercalates with nucleic acids, and MitoTracker Deep Red, which is retained in metabolically active mitochondria, and parasitaemia at the end of 72 h of incubation was quantified via flow cytometry on a BD Accuri C6 cytometer. **B** Dot plots are shown for four different concentrations of TCMDC-137476 (62.5, 15.62, 3.9 and 0 nM) at the end of incubation. Asynchronous parasites were incubated with the compounds (0.0019–2 μM) with an initial parasitaemia of 0.3% at a haematocrit of 2%, and survival curves (dose–response) obtained for the best compounds from the TCAMS library are shown for (**C**) TCMDC-137476, (**D**) TCMDC-142311, (**E**) TCMDC-140136, (**F**) TCMDC-138003 and (**G**) TCMDC-139482. **H** Bar graph showing the respective IC_{50} values ± standard error of the mean identified for each compound. Parasitaemia values were tabulated in Prism-GraphPad software to construct survival curves, and the IC_{50} values were subsequently calculated. The experiments were performed with three independent biological replicates in triplicate. The error bars represent the SEM

Table 2 Selectivity index (SI) between the cytotoxicity of compounds against HEK293 mammalian cells and their antimalarial activities

Code TCMDC	CC_{50} nM HEK293	IC_{50} nM 3D7	Selectivity Index
TCMDC-137476	677.6 nM	15.21 nM	*SI = 44.55
TCMDC-140136	1938 nM	23.56 nM	*SI = 82.26
TCMDC-139867	1279 nM	57.43 nM	*SI = 22.27
TCMDC-138317	16.61 nM	80.79 nM	*SI = 0.20
TCMDC-137905	5552 nM	91.13 nM	*SI = 60.92

The CC_{50} and IC_{50} values for each of the compounds are presented in nanomolar concentrations

* SI. The selectivity index was calculated as the ratio between the cytotoxicity of the compound and its antimalarial activity: $SI = CC_{50}/IC_{50}$

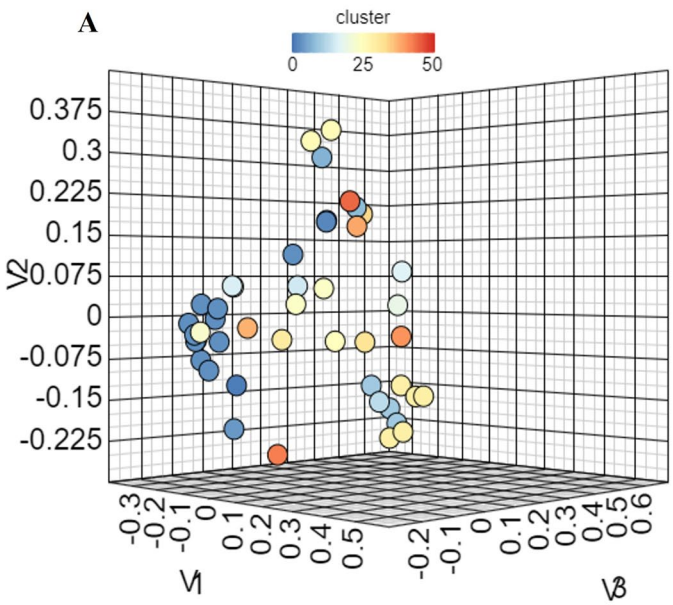
lower than the IC_{50} value of 80.79 nM observed for *P. falciparum*.

Chemoinformatic analysis of the compounds selected from the TCAMS library

The TCAMS library was transferred onto the ChemMine Tools platform by inserting 45 compounds that showed antimalarial activity in the test strip. Using the TCMDC codes and their respective SMILES, the compounds were inserted into the ChemMine platform. Once the library was created in the virtual environment, the tools offered by the platform to perform different analyse were used.. As a first step, the JoeLib tool to process the compounds were selected, this tool calculates physicochemical descriptors for compounds in the workbench via the JoeLib library.

The data generated by the analysis to group the compounds into clusters, as shown in Fig. 2 were used. Similarity cutoff: 0.5; dimension: 3D. Clustering divides the compounds into compartments, with neighbouring compounds defined as those with a similarity greater than the defined cutoff (0.5).

As a result of the cheminformatics analyses performed, the compounds were grouped by structural and physicochemical similarities into different clusters according to their respective similarities. In total, 6 clusters were obtained (2, 3, 10, 24, 26 and 29) in which a total of 25 compounds and 20 (1, 4, 7, 8, 13, 15, 16, 17, 18, 19, 23, 25, 30, 32, 34, 38, 39, 41, 43 and 45) singletons were grouped. Among the clusters, only 2 (3 and 29) consisted of a significant number of compounds, 11 and 5, respectively; clusters 2, 24, and 26 were formed by combining only two compounds, and cluster 10 consisted of 3 compounds.



B

	cluster		cluster
TCMDC-137476	1	TCMDC-139502	17
TCMDC-142311	2	TCMDC-136171	18
TCMDC-142310	2	TCMDC-131917	19
TCMDC-140136	3	TCMDC-137541	23
TCMDC-139482	3	TCMDC-141946	24
TCMDC-139860	3	TCMDC-140381	24
TCMDC-139803	3	TCMDC-139258	25
TCMDC-134281	3	TCMDC-133018	26
TCMDC-140219	3	TCMDC-132764	26
TCMDC-139494	3	TCMDC-135459	29
TCMDC-139786	3	TCMDC-135267	29
TCMDC-139813	3	TCMDC-135301	29
TCMDC-139856	3	TCMDC-135435	29
TCMDC-139634	3	TCMDC-135385	29
TCMDC-138003	4	TCMDC-135829	30
TCMDC-132918	7	TCMDC-134778	32
TCMDC-139867	8	TCMDC-140590	34
TCMDC-135346	10	TCMDC-141151	38
TCMDC-135338	10	TCMDC-124574	39
TCMDC-135344	10	TCMDC-123874	41
TCMDC-125636	13	TCMDC-131795	43
TCMDC-138317	15	TCMDC-133589	45
TCMDC-137905	16		

Fig. 2 Cluster multidimensional scaling (MDS) of the TCAMS compounds. Multidimensional scaling places compounds on a 3D plane so that the distances between points on the plane can preserve the dissimilarities between compounds. The similarity cutoff (≥ 0.5) is used for coloring purposes only. **A** Three-dimensional MDS of the TCAMS library; **B** labelling of compounds in their clusters or isolated elements (singletons)

In cheminformatics data mining, the ancillary data from the original publication of the TCAMS library to identify possible hypothetical targets for each compound studied here were revisited To make the identification of possible targets more accessible in the search for new antimalarial drugs, Gamo et al. [16] identified a total of 4,205 compounds within the TCAMS library with literature annotations on their activity in biochemical assays against human or microbial targets. Clique ou toque aqui para inserir o texto.

Concerning the hypothetical targets of the set of compounds studied here, of the 45 compounds that showed antimalarial activity at the concentrations tested (0.0019–2 μM), 27 were assigned hypothetical targets. Table 3 shows the hypothetical targets described in the work of Gamo et al. [16] and the partitioning of each compound into its respective cluster as determined by multidimensional scaling (MDS).

Among the 27 compounds with identified hypothetical targets, 15 are designated as agonists (TCMDC-139482, TCMDC-139634, TCMDC-139786, TCMDC-139803, TCMDC-139813, TCMDC-140136, and TCMDC-140219) or antagonists (TCMDC-142310, TCMDC-142311, TCMDC-139860, TCMDC-135267, TCMDC-135301, TCMDC-135385, TCMDC-135435 and TCMDC-135459) of serotonin receptors, and all 15 compounds are grouped into clusters 2, 3 and 29, showing a close correlation between structure and hypothetical target. Ten compounds (TCMDC-135338, TCMDC-135344, TCMDC-135346, TCMDC-125636, TCMDC-138317, TCMDC-136171, TCMDC-140381, TCMDC-141946, TCMDC-141151 and TCMDC-123874) are designated peptide hormone receptor antagonists, and only 2 compounds, TCMDC-134281 and TCMDC-133589, have other hypothetical targets, which are adrenergic receptors and Ser/Thr protein kinases, respectively.

Evaluation of compounds related to the human serotonin receptor in *P. falciparum* parasitaemia against the action of the neurotransmitter serotonin and the hormone melatonin

Although the *P. falciparum* parasite does not have proteins in its genome with sequences homologous to those of mammalian serotonin receptors, the action of compounds targeting serotonin receptors has already been documented concerning their antimalarial activity [30–32]. To continue the studies, compounds that hypothetically target serotonin receptors (5-hydroxytryptamine (5HT) agonists and antagonists), were selected, which comprise 3 of the 6 clusters identified after grouping.

To evaluate the action of compounds related to serotonin receptors, an experimental design based on published work were carried out [33–35], which investigated the effects of synthetic compounds derived from indoles on the mechanism of action of the host hormone melatonin in the erythrocytic cycle of *P. falciparum*. Initially, the compounds listed in Table 3, which have serotonin receptors as their hypothetical targets: TCMDC-139482, TCMDC-139634, TCMDC-139786, TCMDC-139803, TCMDC-139813, TCMDC-140136, TCMDC-140219, TCMDC-142310, TCMDC-142311, TCMDC-139860, TCMDC-135267, TCMDC-135301, TCMDC-135385, TCMDC-135435, and TCMDC-135459 were evaluated.

The group of compounds presented varying IC_{50} values ranging from 16.09 ± 5.87 nM (TCMDC-142311) to 1210 ± 314.6 nM (TCMDC-135385). The survival curves and corresponding IC_{50} values for this group of compounds are shown in Fig. 3. For the competition experiments, the compounds were used at a single concentration of 2.5 nM and incubated with asynchronous *P. falciparum* 3D7 cultures (1% parasitaemia, 2% haematocrit) for 48 h.

A concentration of 2.5 nM was chosen for the experiments after three different concentrations were tested (1, 2.5, and 5 nM). A low concentration, which is outside the compound's therapeutic (antimalarial) range, should be used in this test, as no reduction in parasitaemia by the compound should be observed. Serotonin and melatonin were tested at concentrations of 250 nM and 100 nM, respectively, as used in the studies above. After incubation of the samples (48 h) in the presence or absence of serotonin or melatonin, parasitaemia was quantified via flow cytometry, as previously described, for screening the compounds.

As expected, the neurotransmitter serotonin and the hormone melatonin significantly increased *P. falciparum* parasitaemia by 11.86% and 15.05%, respectively, compared with the control, which was incubated with the solvent DMSO 0.075% (v/v). Figure 4 shows the effects of incubation with 2.5 nM serotonin, 250 nM serotonin, and 100 nM melatonin on *P. falciparum* parasitaemia.

The compounds were then incubated with the neurotransmitter serotonin at 250 nM (Fig. 5A) or the host hormone melatonin at 100 nM (Fig. 5B). These experiments were performed as described previously, maintaining the same concentration of compounds (2.5 nM) incubated with serotonin or melatonin to investigate the effects on *P. falciparum* parasitaemia. Several compounds exhibited similar activities to serotonin or melatonin alone. However, no compound demonstrated a significant synergistic effect on parasitaemia.

Concerning the effect of serotonin on parasitaemia, some compounds were able to inhibit the increase in parasitaemia (TCMDC-139634, TCMDC-140219, TCMDC-142310, TCMDC-142311 and TCMDC-135267),

Table 3 Clusters and hypothetical targets of the compounds selected from the TCAMS library

Cluster	Code TCMDC	Hypothetical target*
1	TCMDC-137476	–
2	TCMDC-142310	Serotonin receptor antagonist
	TCMDC-142311	Serotonin receptor antagonist
3	TCMDC-134281	Adrenergic receptor antagonist
	TCMDC-139482	Serotonin receptor agonist
	TCMDC-139494	–
	TCMDC-139634	Serotonin receptor agonist
	TCMDC-139786	Serotonin receptor agonist
	TCMDC-139803	Serotonin receptor agonist
	TCMDC-139813	Serotonin receptor agonist
	TCMDC-139856	–
	TCMDC-139860	Serotonin receptor antagonist
	TCMDC-140136	Serotonin receptor agonist
	TCMDC-140219	Serotonin receptor agonist
4	TCMDC-138003	–
7	TCMDC-132918	–
8	TCMDC-139867	–
	TCMDC-135338	Peptide hormone receptor antagonist
10	TCMDC-135344	Peptide hormone receptor antagonist
	TCMDC-135346	Peptide hormone receptor antagonist
13	TCMDC-125636	Peptide hormone receptor antagonist
15	TCMDC-138317	Peptide hormone receptor antagonist
16	TCMDC-137905	–
17	TCMDC-139502	–
18	TCMDC-136171	Peptide hormone receptor antagonist
19	TCMDC-131917	–
23	TCMDC-137541	–
24	TCMDC-140381	Peptide hormone receptor antagonist
	TCMDC-141946	Peptide hormone receptor antagonist
25	TCMDC-139258	–
26	TCMDC-132764	–
	TCMDC-133018	–
29	TCMDC-135267	Serotonin receptor antagonist
	TCMDC-135301	Serotonin receptor antagonist
	TCMDC-135385	Serotonin receptor antagonist
	TCMDC-135435	Serotonin receptor antagonist
	TCMDC-135459	Serotonin receptor antagonist
30	TCMDC-135829	–
32	TCMDC-134778	–
34	TCMDC-140590	–
38	TCMDC-141151	Peptide hormone receptor antagonist
39	TCMDC-124574	–
41	TCMDC-123874	Peptide hormone receptor antagonist
43	TCMDC-131795	–
45	TCMDC-133589	Ser/Thr Protein Quinase

The compounds in their clusters or isolated elements (singletons) were determined by MDS (3D) clustering

* Hypothetical targets were obtained from the supplemental file (Table 1) of Gamo et al., 2010

showing a degree of significant difference similar to that of the control group treated with DMSO ($P \leq 0.0001$). The levels of the compounds TCMDC-140136, TCMDC-135301, TCMDC-135385, TCMDC-135435, and TCMDC-135459 did not significantly differ from those in the group treated with 250 nM serotonin. Thus, this group of compounds was unable to prevent the effect of the neurotransmitter on *P. falciparum* parasitaemia. The other compounds, TCMDC-139482, TCMDC-139786, TCMDC-139803, TCMDC-139813, and TCMDC-139860, partially inhibited the increase in parasitaemia promoted by serotonin.

Concerning the hormone melatonin, the compounds TCMDC-139634, TCMDC-139786 and TCMDC-142311 could inhibit the increase in parasitaemia promoted by melatonin thoroughly. The effects of the TCMDC-135385 and TCMDC-135459 did not differ from that of melatonin alone and were, therefore, unable to block their effects on the erythrocyte cycle. The other compounds (TCMDC-139482, TCMDC-139803, TCMDC-139813, TCMDC-140136, TCMDC-140219, TCMDC-142310, TCMDC-139860, TCMDC-135267, TCMDC-135301, and TCMDC-135435) were able to inhibit the increase in parasitaemia partially.

Evaluation of TCMDC-135267 effects on *P. falciparum* at erythrocytic cycle

An important question is whether the serotonergic compounds act at a specific time in the *P. falciparum* erythrocytic cycle and whether they cause arrest or direct parasite toxicity. Among the compounds that antagonize the effect of serotonin on *P. falciparum* parasitaemia, TCMDC-139634, TCMDC-140219, TCMDC-142310, TCMDC-142311, and TCMDC-135267 were prominent. Notably, TCMDC-135267 was the most effective at inhibiting the proliferative effects of serotonin (Fig. 5A) and was also effective in preventing the proliferative effects of melatonin (Fig. 5B). Therefore, a flow cytometry approach that can distinguish the discrete erythrocytic cycle stages of the parasite was used to investigate whether TCMDC-135267 interferes directly with *P. falciparum* progression through the ring, trophozoite, and schizont stages over a 48-h period in cultures synchronized to the ring phase with sorbitol (Fig. 6A–D). TCMDC-135267 (150 nM), chloroquine (150 nM) or the DMSO solvent control were added 12 h after synchronization, during the trophic phase. After 12 h further incubation there was no reduction in parasitaemia with either agent, which were not different from the control (Fig. 6E). However,

the parasites were arrested and did not progress through the next cycle of merozoite release and reinvasion that was observed in the controls at 36 and 48 h. TCMDC-135267 was as effective as chloroquine (36 h time point, $p=0.011$ and 0.0053, respectively by 2-way ANOVA, $n=3$). Comparison of the development of each individual erythrocytic cycle phase after 12 h of TCMDC-135267 and chloroquine exposure indicated that both agents slowed the progression of *P. falciparum* growth (fewer trophozoites and schizonts) (Fig. 6F–G). At the 36 h time point parasitaemia increased in the controls ($5.74 \pm 1.7\%$ parasitaemia) and there was a large increase in ring phase parasites ($3.46 \pm 1.0\%$ parasitaemia) to TCMDC-135267 and chloroquine, in which no increase in parasitaemia ($1.18 \pm 0.42\%$, $p=0.0053$, $1.55 \pm 0.87\%$, $p=0.010$, respectively, 2 way ANOVA $n=3$) or ring phase parasites was observed ($0.44 \pm 0.14\%$, $0.30 \pm 0.17\%$, respectively p values both < 0.0001, 2 way ANOVA $n=3$). The parasites continued to proliferate and develop through the erythrocytic cycle in controls, but remained blocked by both drugs at the 48 h time point.

The effect of addition of TCMDC-135267 added at 0 h (ring and early trophozoites phase) and 24 h (mature trophozoite) was also examined and compared to control and chloroquine (Fig. 6H–I). Importantly, the progression from 0 to 12 h did not differ between treatment groups, indicating that the transition from ring to trophozoite phase was not impaired. However, at the 24 h time point in control samples 18% of parasites had developed into schizonts compared to less than 0.5% in both drug-treated groups and no further progression was observed in these cultures at 36 and 48 h. When TCMDC-135267 and chloroquine were added 24 h after synchronization, no change in parasitaemia or erythrocytic cycle phase was observed, but the drug-treated parasites once again failed to proliferate further at the 48 h time point. Taken together, the erythrocytic cycle analysis data provide compelling evidence that TCMDC-135267 prevents the progression from trophozoite to mature schizonts, but does not affect the ability of mature schizonts to egress and invade RBCs. Moreover, TCMDC-135267 mimics the effect of chloroquine in arresting *P. falciparum* growth.

Comparative study of the activity of selected compounds from the TCAMS library in the *P. falciparum* 25 serpentine receptor knockout strain

In an attempt to elucidate the mechanisms of action of the compounds in the TCAMS library, in addition to the previously presented investigations, experiments using a *P. falciparum* strain with the serpentine receptor 25 ($PfSR25^{-}$) knocked out were conducted. The rationale for these experiments is that previous work has shown that the knockout strain has greater susceptibility to antimalarial compounds, especially those targeting the digestive vacuole of the parasite [28, 36, 37]. To investigate the compounds in the TCAMS library, 10 compounds from the library that had IC_{50} values of less than 200 nM for *P. falciparum* 3D7 were selected.

Thus, compounds TCMDC-137476, TCMDC-140136, TCMDC-138003, TCMDC-139482, TCMDC-139860, TCMDC-139867, TCMDC-139803, TCMDC-137905, TCMDC-131917, and TCMDC-139494 were simultaneously evaluated for their respective IC_{50} values. Figure 7 shows the representative dose–response survival curves for 3D7 and parasites evaluated at the end of incubation (72 h) with different concentrations of the compounds (0.0019–2 µM). The results of the comparative screening revealed that none of the compounds tested were significantly different between the two strains, indicating that they do not share a common mechanism of action with PfSR25 knockout.

Discussion

Despite progress in recent decades, resistance to existing antimalarial drugs and the complexity of the *Plasmodium* parasite life cycle highlights the urgency of developing new effective therapies. Resistance to widely used antimalarial drugs such as chloroquine and artemisinin has spread in several endemic regions [38, 39]. This resistance creates a therapeutic gap, compromising the effectiveness of existing treatments and highlighting the need to identify new compounds that can overcome these challenges.

The genomic diversity of the parasite complicates the search for therapeutic targets [40, 41]. New drugs must be able to efficiently target different strains of the parasite, taking into account their genetic variability to ensure

(See figure on next page.)

Fig. 3 Survival curves for TCAMS compounds hypothetically targeting serotonin receptors. Asynchronous parasites were incubated with the compounds TCMDC-139482, TCMDC-139634, TCMDC-139786, TCMDC-139803, TCMDC-139813, TCMDC-140136, TCMDC-140219, TCMDC-142310, TCMDC-142311, TCMDC-139860, TCMDC-135267, TCMDC-135301, TCMDC-135385, TCMDC-135435 and TCMDC-135459 (0. 0019–2 µM), with an initial parasitaemia of 0.3% at a haematocrit of 2%. Parasitaemia at the end of 72 h of incubation was quantified via flow cytometry, and the data generated by the cytometer were analysed via FlowJo V10.9.0 software. Parasitaemia values were tabulated in Prism GraphPad software to construct survival curves. IC_{50} values were first calculated in µM and then converted to nM and are presented above the survival curves for each compound. The experiments were performed in three independent biological replicates. The error bars represent the SEM

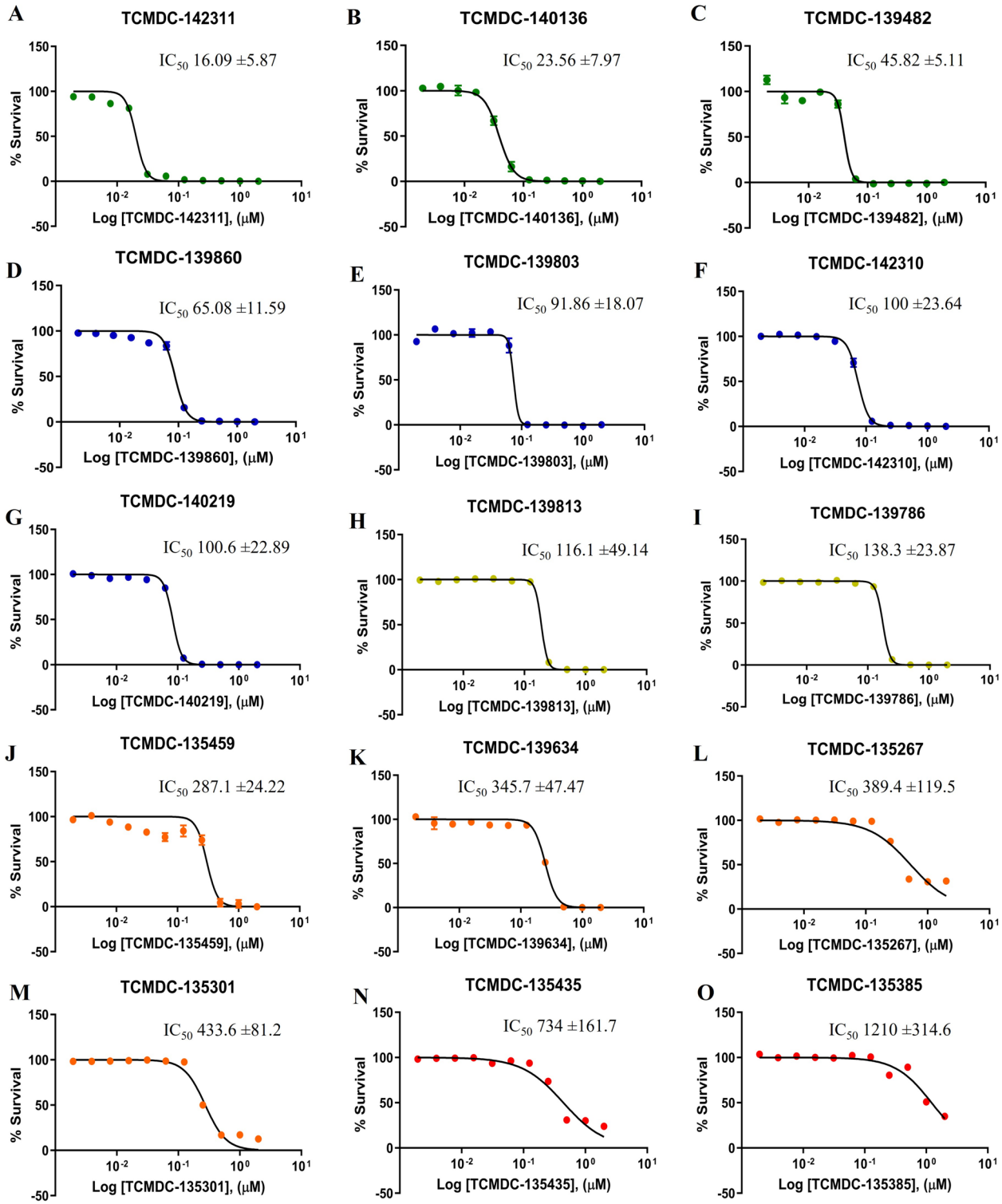


Fig. 3 (See legend on previous page.)

overall efficacy. Recent research has explored innovative approaches to antimalarial drug development. The use of techniques such as genetic engineering to create genetically modified parasites that are susceptible to certain compounds offers promising prospects [42, 43].

The development of new antimalarial drugs requires a collaborative approach that integrates the expertise

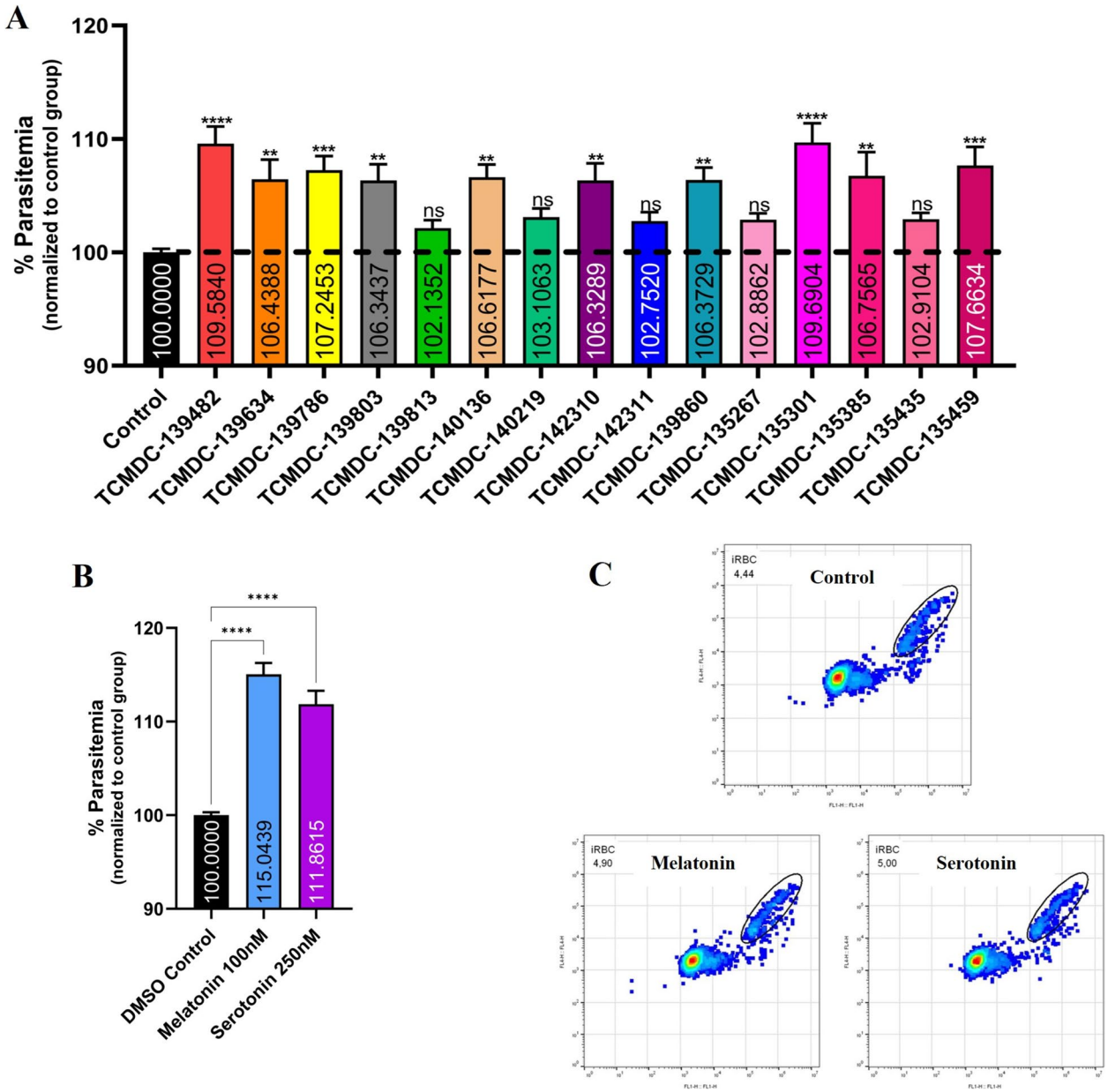


Fig. 4 Effects of TCAMS compounds hypothetically targeting serotonin receptors on *P. falciparum* parasitaemia. **A** Erythrocytes infected with *P. falciparum* 3D7 were incubated for 48 h with 2.5 nM of each compound. **B** Melatonin and serotonin were incubated at 100 nM and 250 nM, respectively. After incubation, the parasites were stained with SYBR Green-I and MitoTracker Deep Red, and finally, parasitaemia was determined via flow cytometry via an Accuri C6 cytometer (BD Bioscience). **C** Dot plots, similar to those shown for the control group, melatonin and serotonin were analysed via FlowJo software. The data represent the percentage of parasitaemia normalized to the control group treated with DMSO (0.075% v/v). The experiments were performed in triplicate in 3 independent replicates. The error bars represent the SEM. "*" indicates a significant difference compared with the control group as determined by one-way ANOVA followed by Dunnett's multiple comparison test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001)

of experts, researchers, academic institutions and the pharmaceutical industry [13–15]. Effective collaborations, such as the launch of the TCAMS library and the Medicines for Malaria Venture initiative, can accelerate drug discovery, clinical trials, and regulatory approval, ensuring faster delivery of innovative treatments to affected populations.

Although the present study used less than 0.5% of the total number of compounds in the TCAMS library, the results provide valuable information because the IC_{50}

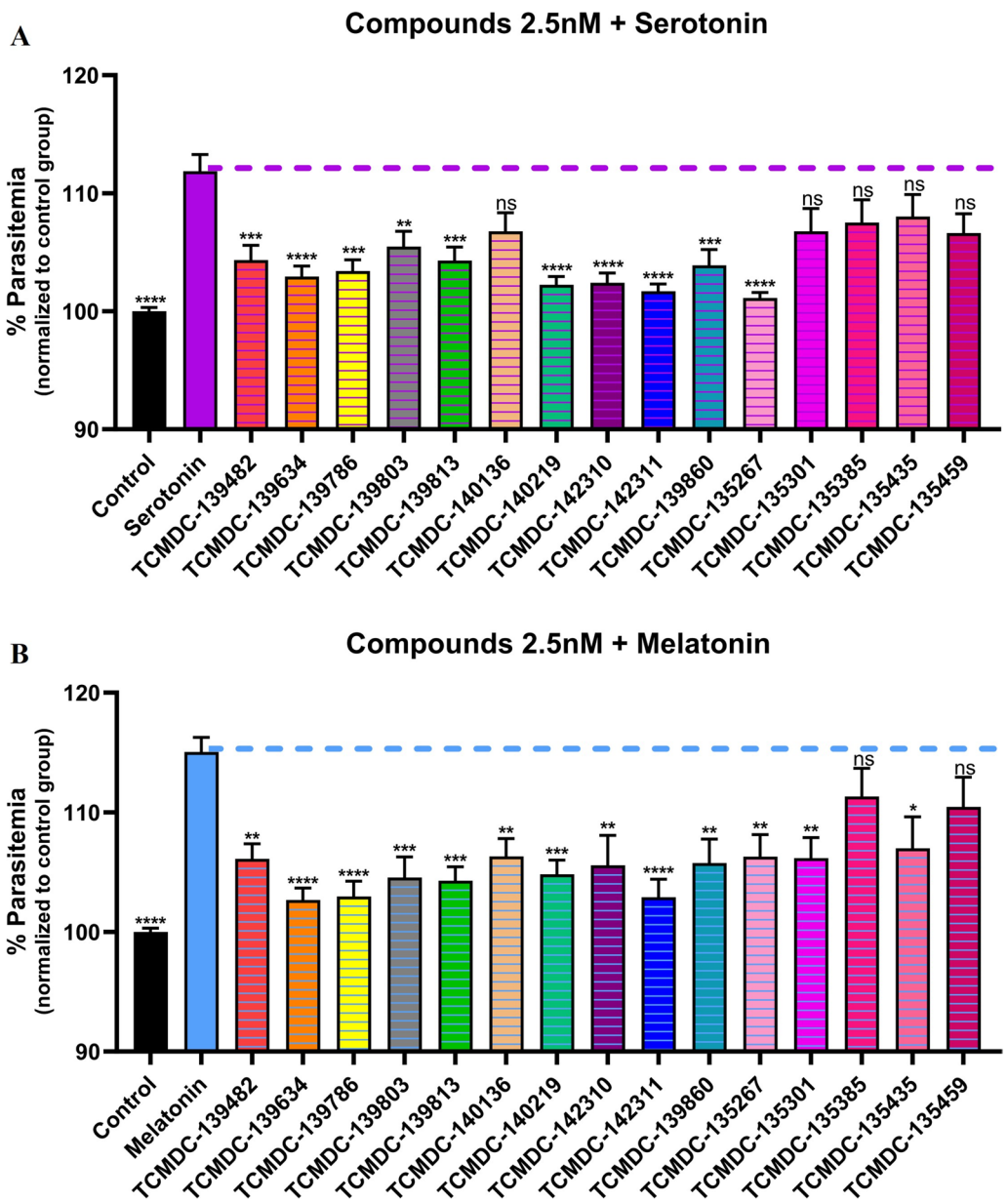


Fig. 5 Effects of TCAMS compounds targeting serotonin receptors on the action of serotonin in the *P. falciparum* cell cycle. Erythrocytes infected with *P. falciparum* 3D7 were incubated for 48 h with 2.5 nM of each of the compounds together with (**A**) 250 nM serotonin or (**B**) 100 nM melatonin. Parasites were stained with SYBR Green-I and MitoTracker Deep Red, and finally, parasitaemia was determined after incubation by flow cytometry via an Accuri C6 cytometer (BD Bioscience). The data represent the percentage of parasitaemia normalized to the control group treated with DMSO (0.075%). The experiments were performed in triplicate in 3 independent replicates. The error bars represent the SEM. "*" indicates a significant difference with respect to treatment with serotonin or melatonin, as determined by one-way ANOVA followed by Dunnett's multiple comparison test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001)

values via a more robust and accurate method than the one used initially, were evaluated here compared with when the library was launched in 2010 [16]. With this analysis, promising hits were identified, such as compounds TCMDC-137476, TCMDC-142311, TCMDC-140136, TCMDC-138003 and TCMDC-139482, especially TCMDC-137476 (IC_{50} 15. 21 ± 5.97 nM) and TCMDC-142311 (IC_{50} 16.09 ± 5.87 nM), which have very competitive IC_{50} values similar to those of the antimalarial drugs used for the clinical treatment of malaria [28].

TCMDC-137476 is structurally similar to the antimalarial quinacrine/mepacrine and has activity in the

sexually mature stage of the parasite. Almela et al. [44] reported that TCMDC-137476 has an IC_{50} of 1.88 µM against *P. falciparum* gametocytes. Other compounds evaluated here, such as TCMDC-140136 and TCMDC-137905, are also active against the sexual forms of the *P. falciparum* parasite, with IC_{50} values in gametocytes of 0.20 µM and 0.23 µM, respectively [44]. When tested against the multidrug-resistant strain of *P. falciparum* Dd2, 41 compounds, with the exception of TCMDC-139860, TCMDC-137541, TCMDC-139856, TCMDC-141151, TCMDC-133589, TCMDC-132496, and TCMDC-140381, were able to inhibit parasite development by more than 50% at a concentration of 2 µM [16]. In particular, the 5 most potent hits identified here (TCMDC-137476, TCMDC-142311, TCMDC-140136, TCMDC-138003 and TCMDC-139482) inhibited the resistant strain by more than 90% [16].

Drug discovery is a complex process that necessitates a thorough evaluation of various parameters, including therapeutic efficacy and safety. Cytotoxicity, defined as the ability of a compound to damage cells, is a crucial consideration in this context [45, 46]. In these studies, the cytotoxicity evaluation of TCAMS compounds in human embryonic kidney cells (HEK293) supports the findings of Gamo et al. [16], suggesting the safety of these compounds for in vivo studies.

After characterizing the respective IC_{50} values for each compound and assessing its cytotoxicity, the antimalarial mechanism of action and attempt to identify hypothetical targets were studied. According to Cabrera [47], the need to identify the target is not essential in the early stages of the development of phenotypic projects; generally, this identification occurs later, in conjunction with the generation of resistant mutants. However, the discovery of the target of a specific compound can accelerate the research stages and increase the safety of these processes.

Approximately 70% of the targets identified for the TCAMS library are G protein-coupled receptors (GPCRs) or protein kinases, reflecting the relative abundance of the different ligand classes in the library. Many compounds, including GPCRs, nuclear receptors, ion channels, and transporters, have shown activity against targets that do not have orthologs in the parasite genome [16]. According to the authors, a plausible explanation is that these compounds eliminate parasites through interactions with targets unrelated to those found for human proteins [16, 47]. However, one should also consider the hypothesis that *P. falciparum* possesses essential proteins structurally and functionally similar to human targets, even if there is no significant similarity in the primary amino acid sequence.

This study identified hypothetical targets for 27 of the 48 compounds evaluated, with 15 classifieds as serotonin receptor agonists or antagonists. Using a competitive approach, the action of these compounds were assessed. The compounds TCMDC-139482, TCMDC-139634, TCMDC-139786, TCMDC-139803, TCMDC-140136, TCMDC-142310, TCMDC-139860, TCMDC-135301, TCMDC-135385, and TCMDC-135459 induced a significant increase in parasitaemia, particularly TCMDC-139482 (9.58%) and TCMDC-135301 (9.69%), which led to increases similar to those observed with serotonin. In contrast, low concentrations (2.5 nM) of TCMDC-139813, TCMDC-140219, TCMDC-142311, TCMDC-135267, and TCMDC-135435 did not significantly affect parasitaemia compared with the control.

The TCMDC-139634, TCMDC-140219, TCMDC-142310, TCMDC-142311, and TCMDC-135267 act as antagonists of the serotonin pathway. In contrast, TCMDC-139634, TCMDC-139786, and TCMDC-142311 acted as antagonists of the melatonin pathway, with statistical values equal to those of the control group ($P \leq 0.0001$). TCMDC-135267, TCMDC-139634 and TCMDC-142311 were potent blockers of the effects of serotonin and melatonin on the parasite erythrocyte life cycle. Considering the effects of the compounds in the presence and absence of serotonin or melatonin, TCMDC-139634, TCMDC-139786, and TCMDC-142310 appear to be potential partial agonists of this pathway in the parasite, as they increase parasitaemia in the 3D7 strain while also inhibiting the effects of serotonin and melatonin. In contrast, TCMDC-140219, TCMDC-142311, and TCMDC-135267 appear to be potential full antagonists of the pathway, as they do not

(See figure on next page.)

Fig. 6 Effect of TCMDC-135267 on the erythrocytic cycle of *P. falciparum*. *P. falciparum* cultures were synchronized with sorbitol (Time 0) and plated at 1% parasitaemia, 2% haematocrit. Samples were taken every 12 h and erythrocytic cycle phases and parasitaemia were determined via flow cytometry. **A–D** Normal life cycle progression measured in the presence of vehicle control (0.1% DMSO). **E–F** Effect of chloroquine (CQ, 150 nM) and TCMDC-135267 (150 nM) on *P. falciparum* progression when added at Time 12 h (Trophozoite phase) and measured over the subsequent 48 h period. Data are mean ± SD of 3 independent experiments. Summary data show, (**E**) total parasitaemia (**F**) parasitaemia and the distribution of parasites in each erythrocytic cycle phase, and (**G**) percentage of parasites in each erythrocytic cycle phase (note total parasitaemia in center of pie charts). **H-I** Effect of chloroquine and TCMDC-135267 on *P. falciparum* cell cycle progression when added at Time 0 or Time 24 (Trophozoite phase) and measured over a 48 h period. Data are mean ± SD from a single experiment performed in triplicate. Summary data shows (**H**) parasitaemia and the distribution of parasites in each erythrocytic cycle phase, and (**I**) the percentage of parasites in each erythrocytic cycle phase.

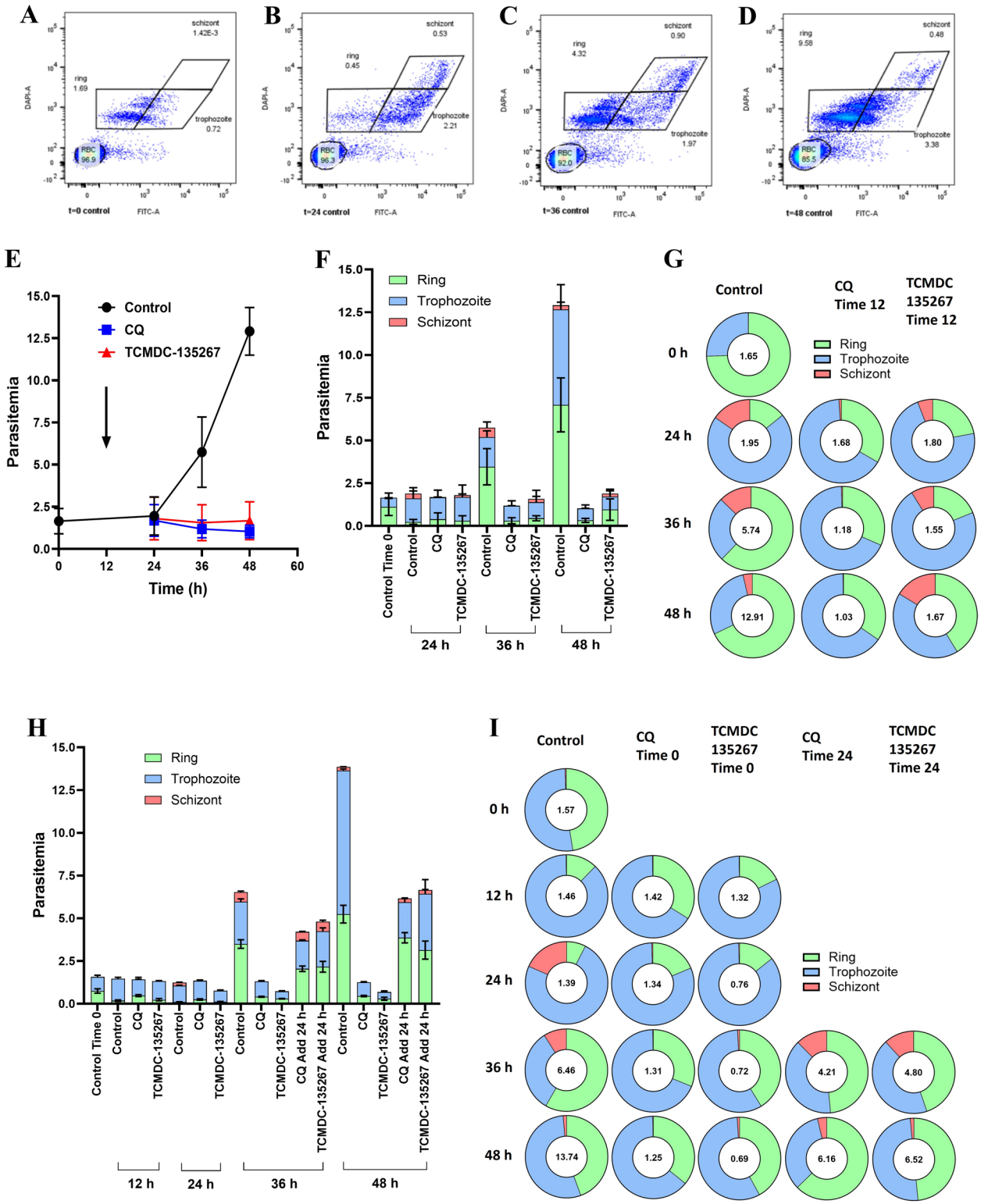


Fig. 6 (See legend on previous page.)

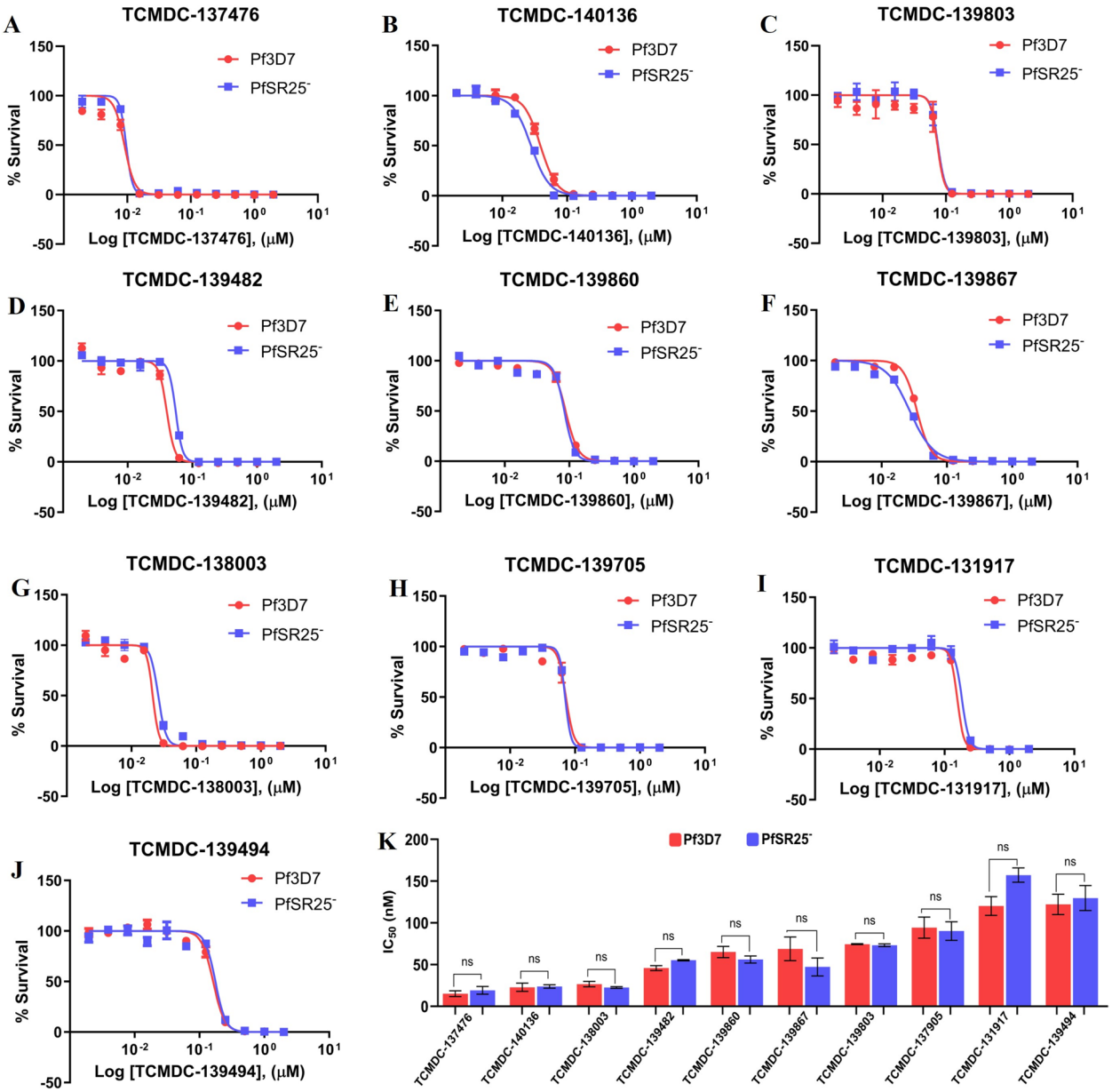


Fig. 7 Determination of the in vitro antimalarial activity of the TCAMS compounds selected for testing on *P. falciparum* 3D7 and PfSR25⁻. Representative survival curves for the compounds selected from the TCAMS library. **A** TCMDC-137476, (**B**) TCMDC-140136, (**C**) TCMDC-138003, (**D**) TCMDC-139482, (**E**) TCMDC-139860, (**F**) TCMDC-139867, (**G**) TCMDC-139803, (**H**) TCMDC-137905, (**I**) TCMDC-131917, and (**J**) TCMDC-139494 for the 3D7 (red) and PfSR25⁻ (blue) strains. Asynchronous parasites were incubated with the test compounds (0.0019–2 μM) with an initial parasitaemia of 0.3% at a haematocrit of 2%. Parasitaemia at the end of 72 h of incubation was quantified via flow cytometry, and the data generated were analysed via FlowJo V10.9.0 software. Parasitaemia values were tabulated in Prism-GraphPad software to construct survival curves, and the IC_{50} values were subsequently calculated. **K** The IC_{50} values (± standard error of the mean) for both strains (3D7 and PfSR25⁻) are presented in a bar graph, and significant differences were determined by Student's t test (ns = not significant). The error bars represent the SEMs. The experiments were performed in triplicate with three independent biological replicates

increase parasitaemia in the presence or absence of serotonin or melatonin.

Although serotonin or melatonin receptors have not yet been identified in the *P. falciparum* parasite, serotonin receptor agonists and antagonists are promising antimalarial candidates. For example, dihydroergotamine methanesulfonate, a serotonin receptor antagonist, has been correlated with, interacts with, or is transported by PfMDR1, a transporter involved in antimalarial drug resistance [48]. The serotonin receptor agonist

8-OH-DPAT (8-hydroxy-N-(di-n-propyl)-aminotetralin) showed antimalarial activity with an IC_{50} value of 0.4 µM, and patch clamp assays suggested that this agonist acts by blocking a membrane channel in parasitized erythrocytes [49]. The indole ring derivative Melatotosil blocks the action of melatonin in the cell cycle of *P. falciparum* 3D7. It is described as a partial agonist of the melatonin pathway in the parasite [33].

Comparative screening with different parasite strains is crucial for dissecting the mechanism of action and the possible involvement of proteins in the efficacy of antimalarial drugs [28]. For *P. falciparum* serpentine receptor 25, exposure of knockout parasites ($PfSR25^{-}$) to chloroquine for 24 h significantly reduced parasitaemia compared with that of wild-type parasites (3D7), suggesting that it somehow counteracts the antimalarial effects of chloroquine [36]. Compared with the control (solvent-treated) and wild-type parasite treatments, the antimalarial drug piperaquine, when administered to trophozoites for 2 h, significantly reduced the size of the haemozoin polymer [36]. Comparative compound screening with PfSR25 and 3D7 revealed that the knockout strain was more sensitive to synthetic compounds derived from 1 and 2*H* 1,2,3-triazole regioisomers [37]. Additionally, a 2021 study reported that $PfSR25^{-}$ exhibited increased susceptibility to the antimalarial drugs lumefantrine and piperaquine, which target haemozoin metabolism [28]. A previous study revealed that piperaquine, like lumefantrine, can decrease *pfsr25* gene expression in ring-stage parasites [28, 50]. For the TCAMS compounds evaluated here, no significant difference was observed in the IC_{50} values between the two strains, strongly suggesting that PfSR25 is not involved in the mechanism of action of these compounds.

In summary, the research on the compounds in the TCAMS library significantly contributes to investigating new mechanisms of action that will favor the development of new antimalarial drugs. The collaborative approach highlighted is crucial to accelerate the discovery of effective compounds. The results are promising, especially with identifying compounds such as TCMDC-137476 and TCMDC-142311, whose IC_{50} values are remarkably competitive for drugs in clinical use. In addition, the analysis of cytotoxicity and the identification of hypothetical targets provide a solid basis for further studies.

Conclusion

This study was designed to contribute to the existing knowledge base and encourage the scientific community to join forces in the search for more effective and affordable therapies to combat malaria. The IC_{50} characterization of compounds TCMDC-137476, TCMDC-142311, TCMDC-140136, TCMDC-138003, and TCMDC-139482 provides the community with competitive compounds for the pharmaceutical market and sheds light on the future of new antimalarial drug development. Data evaluating the compounds' cytotoxicity and a possible elucidation of their molecular targets can accelerate the in vivo research stages and make these processes safer and more agile.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05271-3.

Additional file 1.

Additional file 2.

Acknowledgements

We thank the pharmaceutical company GlaxoSmithKline (GSK) for the compounds and COLSAN (São Paulo—Brazil) for the blood. The human biological samples were sourced ethically, and their research use was in accordance with the terms of informed consent under an IRB/EC approved protocol. The project was approved by the Research Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo (FCF-USP), identification number: CAAE 46598621.2.0000.0067.

Author contributions

BMS designed and performed all the experiments, LRCM assisted in conducting the screenings, AL carried out additional screenings, MFP performed previous investigations that led to selecting the set of compounds used. AM conducted control experiments with the antimalarial drug chloroquine. CRSG, APT and FJG are the principal investigators who contributed to the design of the experiments, discussion of the data, and management of the project. BMS, CRSG, PJB, APT and FJG contributed to the article's writing and interpretation of the data.

Funding

This work was supported by a grant from the São Paulo Research Foundation (FAPESP) to the CRSG (Process 2017/08684–7). BMS is a FAPESP fellow (Process 2020/08988–9) and NIH grant NIAID R01 AI109927. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil. [2]Department of Chemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP 05508-000, Brazil. [3]Department of Pharmacology, Physiology & Neuroscience, New Jersey Medical School, Rutgers University, Newark, NJ 07103, USA. [4]Global Health Medicines R&D, GlaxoSmithKline (GSK), Tres Cantos, 28760 Madrid, Spain.

Received: 29 May 2024 Accepted: 21 January 2025
Published online: 14 February 2025

References

1. Levano-Garcia J, Dluzewski AR, Markus RP, Garcia CRS. Purinergic signalling is involved in the malaria parasite *Plasmodium falciparum* invasion to red blood cells. Purinergic Signal. 2010;6:365–72.
2. Gutiérrez-López R, Martínez-De La Puente J, Gangoso L, Soriguer R, Figuerola J. *Plasmodium* transmission differs between mosquito species and parasite lineages. Parasitology. 2020;147:441–7.
3. Sato S. *Plasmodium* - a brief introduction to the parasites causing human malaria and their basic biology. J PhysiolAnthropol. 2021;40:3.
4. WHO. World malaria report 2022. Geneva: World Health Organization; 2022.
5. WHO. World Malaria Report 2023. Geneva: World Health Organization; 2023.
6. Daily JP, Minuti A, Khan N. Diagnosis, treatment, and prevention of malaria in the US: a review. JAMA. 2022;328:460–71.
7. Schlitzer M. Antimalarial drugs – what is in use and what is in the pipeline. Arch Pharm (Weinheim). 2008;341:149–63.
8. Cowell AN, Winzeler EA. The genomic architecture of antimalarial drug resistance. Brief Funct Genomics. 2019;18:314–28.
9. Dhingra SK, Redhi D, Combrinck JM, Yeo T, Okombo J, Henrich PP, et al. A variant PfCRT isoform can contribute to *Plasmodium falciparum* resistance to the first-line partner drug piperaquine. mBio. 2017;8:e00303-17.
10. Nadjm B, Behrens RH. Malaria: an update for physicians. Infect Dis Clin North Am. 2012;26:243–59.
11. Wicht KJ, Mok S, Fidock DA. Molecular mechanisms of drug resistance in *Plasmodium falciparum* malaria. Annu Rev Microbiol. 2020;74:431–54.
12. Blasco B, Di L, Fidock DA. Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. Nat Med. 2017;23:917–28.
13. Ashley EA, Phyo AP. Drugs in development for malaria. Drugs. 2018;78:861–79.
14. Croft SL. Public-private partnership: from there to here. Trans R Soc Trop Med Hyg. 2005;99:9–14.
15. Trouiller P, Olliaro P, Torreele E, Orbinski J, Laing R, Ford N. Drug development for neglected diseases: a deficient market and a public-health policy failure. Lancet. 2002;359:2188–94.
16. Gamo FJ, Sanz LM, Vidal J, De Cozar C, Alvarez E, Lavandera JL, et al. Thousands of chemical starting points for antimalarial lead identification. Nature. 2010;465:305–10.
17. Williamson AE, Ylioja PM, Robertson MN, Avery V, Baell JB, Batchu H, et al. Open source drug discovery: highly potent antimalarial compounds derived from the tres cantos arylpyrroles. ACS Cent Sci. 2016;2:687–701.
18. Rueda L, Castellote I, Castro-Pichel J, Chaparro MJ, De La Rosa JC, Garcia-Perez A, et al. Cyclopropyl carboxamides: a new oral antimalarial series derived from the Tres Cantos Anti-Malarial Set (TCAMS). ACS Med Chem Lett. 2011;2:840–4.
19. Sanz LM, Jiménez-Díaz MB, Crespo B, De-Cozar C, Almela MJ, Angulo-Barturen I, et al. Cyclopropyl carboxamides, a chemically novel class of antimalarial agents identified in a phenotypic screen. Antimicrob Agents Chemother. 2011;55:5740–5.
20. Rottmann M, McNamara C, Yeung BKS, Lee MCS, Zou B, Russell B, et al. Spiroindolones, a potent compound class for the treatment of malaria. Science. 2010;329:1175–80.
21. Calderón F, Vidal-Mas J, Burrows J, De La Rosa JC, Jiménez-Díaz MB, Mulet T, et al. A divergent SAR study allows optimization of a potent 5-HT2c inhibitor to a promising antimalarial scaffold. ACS Med Chem Lett. 2012;3:373–7.
22. Meyers MJ, Tortorella MD, Xu J, Qin L, He Z, Lang X, et al. Evaluation of aminohydantoins as a novel class of antimalarial agents. ACS Med Chem Lett. 2014;5:89–93.
23. Weidner T, Nasereddin A, Preu L, Grünefeld J, Dzikowski R, Kunick C. Novel 2-phenoxyanilide congeners derived from a hit structure of the TCAMS: synthesis and evaluation of their in vitro activity against *Plasmodium falciparum*. Molecules. 2016;21:223.
24. McConville M, Fernández J, Angulo-Barturen Í, Bahamontes-Rosa N, Ballell-Pages L, Castañeda P, et al. Carbamoyl triazoles, known serine protease inhibitors, are a potent new class of antimalarials. J Med Chem. 2015;58:6448–55.
25. Ekland EH, Schneider J, Fidock DA. Identifying apicoplast-targeting antimalarials using high-throughput compatible approaches. FASEB J. 2011;25:3583–93.
26. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976;193:673–5.
27. Backman TWH, Cao Y, Girke T. ChemMine tools: an online service for analyzing and clustering small molecules. Nucleic Acids Res. 2011;39:W486–91.
28. Santos BM, Dias BKM, Nakabashi M, Garcia CRS. The knockout for G protein-coupled receptor-like PfSR25 increases the susceptibility of malaria parasites to the antimalarials lumefantrine and piperaquine but not to medicine for malaria venture compounds. Front Microbiol. 2021;12: 638869.
29. Ndjakou Lenta B, Vonthron-Sénécheau C, Fongang Soh R, Tantangmo F, Ngouela S, Kaiser M, et al. In vitro antiprotozoal activities and cytotoxicity of some selected Cameroonian medicinal plants. J Ethnopharmacol. 2007;111:8–12.
30. Chattopadhyay A, Kalipatnapu S. Serotonin1A receptor agonist acquires an antimalarial connection. J Biosci. 2004;29:1–2.
31. Coles TA, Briggs AM, Hambly MG, Céspedes N, Fellows AM, Kaylor HL, et al. Ingested histamine and serotonin interact to alter *Anopheles stephensi* feeding and flight behavior and infection with *Plasmodium* parasites. Front Physiol. 2023;14:1247316.
32. Montazeri M, Fakhar M, Keighobadi M. The potential role of the serotonin transporter as a drug target against parasitic infections: a scoping review of the literature. Recent Adv AntiInfect Drug Discov. 2022;17:23–33.
33. Dias BKM, Nakabashi M, Alves MRR, Portella DP, dos Santos BM, da Silva Costa, Almeida F, et al. The Plasmodium falciparum eIK1 kinase (PfeIK1) is central for melatonin synchronization in the human malaria parasite Melatotosil blocks melatonin action on parasite cell cycle. J Pineal Res. 2020;69:12685.
34. Mallaupoma LRC, Dias BKM, Singh MK, Honorio RI, Nakabashi M, Kisukuri CM, et al. Decoding the role of melatonin structure on *Plasmodium falciparum* human malaria parasites synchronization using 2-sulfenylindoles derivatives. Biomolecules. 2022;12:638.
35. Schuck DC, Jordão AK, Nakabashi M, Cunha AC, Ferreira VF, Garcia CRS. Synthetic indole and melatonin derivatives exhibit antimalarial activity on the cell cycle of the human malaria parasite *Plasmodium falciparum*. Eur J Med Chem. 2014;78:375–82.
36. Moraes MS, Budu A, Singh MK, Borges-Pereira L, Levano-Garcia J, Currà C, et al. *Plasmodium falciparum* GPCR-like receptor SR25 mediates extracellular K+ sensing coupled to Ca2+ signaling and stress survival. Sci Rep. 2017;7:9545.
37. dos Santos BM, Gonzaga DTG, da Silva FC, Ferreira VF, Garcia CRS. *Plasmodium falciparum* knockout for the GPCR-like PfSR25 receptor displays greater susceptibility to 1,2,3-triazole compounds that block malaria parasite development. Biomolecules. 2020;10:1197.
38. Rosenbaum DM, Rasmussen SGF, Kobilka BK. The structure and function of G-protein-coupled receptors. Nature. 2009;459:356–63.
39. Uwimana A, Legrand E, Stokes BH, Ndikumana JLM, Warsame M, Umulisa N, et al. Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. Nat Med. 2020;26:1602–8.
40. Volkman SK, Sabeti PC, Decaprio D, Neafsey DE, Schaffner SF, Milner DA, et al. A genome-wide map of diversity in *Plasmodium falciparum*. Nat Genet. 2006;39:113–9.
41. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature. 2002;419:498–511.
42. Madhav H, Hoda N. An insight into the recent development of the clinical candidates for the treatment of malaria and their target proteins. Eur J Med Chem. 2021;210: 112955.
43. Na-Bangchang K, Karbwang J. Current status of malaria chemotherapy and the role of pharmacology in antimalarial drug research and development. Fundam Clin Pharmacol. 2009;23:387–409.
44. Almela MJ, Lozano S, Lelièvre J, Colmenarejo G, Coterón JM, Rodrigues J, et al. a new set of chemical starting points with *Plasmodium falciparum* transmission-blocking potential for antimalarial drug discovery. PLoS ONE. 2015;10: e0135139.
45. Niles AL, Moravec RA, Riss TL. Update on in vitro cytotoxicity assays for drug development. Expert Opin Drug Discov. 2008;3:655–69.
46. Slater K. Cytotoxicity tests for high-throughput drug discovery. Curr Opin Biotechnol. 2001;12:70–4.

47. Cabrera AC. Collaborative drug discovery and the Tres Cantos Antimalarial Set (TCAMS). Drug Discov Today. 2019;24:1304–10.
48. Yuan J, Johnson RL, Huang R, Wichterman J, Jiang H, Hayton K, et al. Genetic mapping of targets mediating differential chemical phenotypes in *Plasmodium falciparum*. Nat Chem Biol. 2009;5:765–71.
49. Locher CP, Ruben PC, Gut J, Rosenthal PJ. 5HT1A serotonin receptor agonists inhibit *Plasmodium falciparum* by blocking a membrane channel. Antimicrob Agents Chemother. 2003;47:3806–9.
50. Mwai L, Diriye A, Masseno V, Muriithi S, Feltwell T, Musyoki J, et al. Genome wide adaptations of *Plasmodium falciparum* in response to lumefantrine selective drug pressure. PLoS ONE. 2012;7: e31623.

Publisher's Note