Gametocyte-specific and all-blood-stage transmission-blocking chemotypes discovered from high throughput screening on *Plasmodium falciparum* gametocytes

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Blocking *Plasmodium falciparum* human-to-mosquito transmission is essential for malaria elimination, nonetheless drugs killing the pathogenic asexual stages are generally inactive on the parasite transmissible stages, the gametocytes. Due to technical and biological limitations in high throughput screening of non-proliferative stages, the search for gametocyte-killing molecules so far tested one tenth the number of compounds screened on asexual stages. Here we overcome these limitations and rapidly screened around 120,000 compounds, using not purified, bioluminescent mature gametocytes. Orthogonal gametocyte assays, selectivity assays on human cells and asexual parasites, followed by compound clustering, brought to the identification of 84 hits, half of which are gametocyte selective and half with comparable activity against sexual and asexual parasites. We validated seven chemotypes, three of which are, to the best of our knowledge, novel. These molecules are able to inhibit male gametocyte exflagellation and block parasite transmission through the Anopheles mosquito vector in a standard membrane feeding assay. This work shows that interrogating a wide and diverse chemical space, with a streamlined gametocyte HTS and hit validation funnel, holds promise for the identification of dual stage and gametocyte-selective compounds to be developed into new generation of transmission blocking drugs for malaria elimination.
In 2021, the World Health Organization (WHO) “World malaria report 2021” reported 241 million new malaria cases and over 600,000 deaths, mainly due to *Plasmodium falciparum*, confirming that progresses in controlling malaria, reached between 2000 and 2015, have stalled in the past five years. This led the WHO to adopt a new strategy to accelerate progresses towards the long-term goal of malaria elimination. In fact, malaria elimination is an objective set in the WHO “Global Technical Strategy for Malaria 2016–2030” in the Strategic Framework Pillar to “Accelerate Efforts Toward Elimination” (WHO Global Technical Strategy for Malaria 2016–2030). In malaria, symptoms are caused by the pathogenic asexual stages of the Plasmodium protozoan parasite that infect and proliferate within red blood cells able to adhere to the microvascular endothelium of virtually all internal organs. Instead, transmission of the parasite to *Anopheles* mosquito vectors relies on gametocytes: non-dividing, highly differentiated Plasmodium sexual stages that, in the case of *P. falciparum*, mature in red blood cells in 10–12 days through five morphological stages (I–V). While circulating, mature gametocytes are taken in the mosquito blood meal, in which they transform into male and female extracellular gametes. The mating of the gametes in the mosquito gut generates a fertilized zygote that eventually produces thousands of sporozoites. These are passed on from the insect salivary glands to humans at the mosquito bite.

With the aim of reaching malaria elimination, Medicines for Malaria Venture (MMV) proposed a set of revised target product profiles (TPP) to treat and prevent malaria. Besides the search for new molecules to treat the acute phase of malaria, and to face the constant threat of the onset of parasite drug resistance (target candidate profile 1, TCP1), the focus is put on the discovery of drugs able to prevent infections by blocking parasite transmission from infected individuals to the mosquito vectors (target candidate profile 5, TCP5). This goal can be achieved by compounds targeting the *Plasmodium* gametocytes.

Except for primaquine, which however has safety liabilities as it can cause haemolysis in individuals with glucose-6-phosphate dehydrogenase deficiencies, current antimalarial drugs fail to kill *P. falciparum* mature gametocytes. This is likely due to the different biology underlying sexual vs asexual stage development, suggesting that future anti-gametocyte drugs may have to hit gametocyte specific mechanisms(s) of action. Nevertheless, dual active drugs, ideally active with comparable potency on both stages, are currently considered highly desirable by several malaria drug discovery initiatives, e.g., MMV. Example of compounds being investigated are the spiroindolone Cipargamin/KAE609, targeting the PIATP4 cation ATPase, the 2-Aminopyridine MMV048, targeting the parasite phosphatidylinositol-4-kinase PI4K and the quinoline-4-carboxamide M5717/DDD498, active on the parasite translation/elongation factor. Importantly, potential gametocyte specific drugs will not cause the selection of parasite resistant genotypes during asexual proliferation, as predictable for dual active drugs, thus securing a longer lifetime.

So far, the cumulative amount of compounds tested against *P. falciparum* gametocytes was less than 300,000 in several independent screenings, compared to the over 5 million that were screened against the asexual stage parasites, half of which in only two screenings. This is partly due to the challenge of reliably measuring the viability of non-proliferative gametocytes in a sensitive and robust high throughput screening (HTS) assay format. A variety of HTS gametocyte assays were used so far relying on multiple detection techniques: fluorescent/luminescent reporters, parasite enzymes activity assays, gametocyte ATP content determination, uptake of redox sensitive dyes and the assessment of the motility of male gamete flagella. In these approach, one or more protocol steps are not amenable to large screenings or may affect gametocyte response to treatment. Some examples are: the need to purify gametocytes from uninfected erythrocytes and gametocyte treatment in the absence of human serum and the use of technically demanding imaging procedures. These have altogether prevented to establish screening approaches for anti-gametocyte molecules able to interrogate a chemical space of a size like the one explored on the asexual blood stages.

To this end, here we report the establishment of a simple, sensitive and robust HTS assay funnel for the identification of anti-gametocyte compounds including set of counter-screenings and validation assays. With this tool we set out to identify bona fide gametocyte active chemotypes and investigate the selectivity of them against the human host and the asexual stage parasites.

**Results**

Gametocyte assay optimization and miniaturization. In order to screen the CNCSs (the Italian national chemical collection) compound library for molecules able to inhibit *P. falciparum* gametocyte viability, we optimized and miniaturized an assay using the genetically modified parasite line 3D7elo1-pfs16-CBG99. In this line, the CBG99 luciferase-coding region (from the click beetle *Pyrophorus plagiophthalamus*) is integrated into the parasite genome and expressed under the control of the *pfs16* gametocyte-specific promoter. Gametocytes of this line produce a strong and gametocyte-specific bioluminescent signal with optimal stability. The highest reporter signal is reached in stage IV–V gametocytes, with optimal signal to background (S/B) ratio. According to our previous work, the use in the luciferase assay of a non-lyzing, ATP-free, D-luciferin substrate was adopted to more reliably measure gametocyte viability, as confirmed by single gametocyte bioluminescence imaging.

Obtaining an accurate gametocyte synchronization with minimal parasite handling is of paramount importance to establish a robust and reliable HTS assay. To this aim, the timing of addition of N-acetyl glucosamine (NAG), used to clear residual asexual stages after appearance of stage I gametocytes, was optimized. A three-day treatment with 50 mM NAG, followed by an additional five days of cultivation, yielded cultures with about 2% gametocytaemia of early-stage V gametocytes, as confirmed by microscopy examination of Giemsa-stained blood smears (Fig. 1a). Using these cultures, different haematocrit percentages (0.625, 1.25 and 2.5%) and two incubation times (48- and 72 h) were tested to identify the optimal luciferase readout. Ten µM methylene blue (MB) was used as reference compound (Fig. 1b). As a result, 0.625% haematocrit at 48 h was found to be an acceptable compromise between an excellent signal to background (S/B) ratio (i.e., the ratio between the average of the vehicle treated vs MB treated cultures, a ratio greater than three is commonly considered appropriate, in this case it was 13.6) and a reasonable culture amount per well. Finally, using the optimized assay conditions, we established the final assay metrics and robustness by incubating half of a 384-well plate with 10 µM MB and half 384-well plate with vehicle dimethyl sulfoxide (DMSO) for 48 h, followed by luminescence detection. Results reported in Fig. 1c show that, in these conditions, we obtained a S/B of 53 and a Z’ of 0.76. In addition, the coefficient of variation% (CV%) of both positive and negative controls was less than 10% (Fig. 1c), that is commonly considered the threshold for optimal assay precision. The selected screening conditions not only combine both optimal biological conditions and good readout but are also straightforward because they do not require gametocyte purification steps. Finally, parasite culture volumes necessary to perform an entire screening can be easily managed: a total volume of 100 ml routine gametocyte culture (4% haematocrit, 1–2%
gametocytaemia) was enough for an average screening batch of 19,200 data points. Altogether these results paved the way for the HTS campaign described here.

High throughput screening and hit identification. A collection of 119,059 compounds of the CNCCS library was tested on gametocytes of the 3D7elo1-pfs16-CBG99 strain at 10 µM using the protocol described above. The Z’ values were found to be greater than or equal to 0.5 for all screening plates indicating that the assay was sufficiently robust to test the compounds (Fig. 2a). The distribution of the compound activities converged to normal (or Gaussian) distribution (Fig. 2b); therefore, compounds with an activity equal to or greater than the average activity plus three standard deviations (41% inhibition) were considered hit compounds. Applying these parameters, 960 compounds, corresponding to 0.81% of the total, were identified as active in the primary screening and subjected to confirmation assays.

Hit confirmation. To validate the active compounds the 960 hits were tested at three concentrations, namely 20, 4, and 0.8 µM, on the same 3D7elo1-pfs16-CBG99 strain used for the screening. Furthermore, they were also tested at 10 µM in a mammalian cell

Fig. 1 HTS assay optimization. a Representative image of the gametocyte stages used in the HTS optimization: Giemsa-stained blood smears of infected red blood cell (RBC) cultivation after three-day treatment with 50 mM NAG followed by additional 5 days of cultivation. Early-stage V gametocytes are visible and a 2% gametocytaemia can be calculated. Black scale bar = 10 µm b Luminescence counts, expressed as Relative Light Units (RLU, averages and standard deviations are depicted, n = 4 independent experiments or n = 2 independent experiments for MB), of hematocrit dilutions of uninfected and of vehicle or 10 µM Methylene Blue (MB) treated infected RBC at 48- and 72 h incubation. Cultures were treated with NAG as above described. c Luminescence reporter assay metrics of MB treated vs. untreated culture at 48 h (averages and standard deviations are depicted, n = 48 technical replicates). Coefficient of variation percentage (CV%) are reported within each bar. Assay with a precision of CV% < 10 are considered optimal. For both a and b due to the high difference in magnitude between high and low conditions, a statistical analysis to assess significance was not needed.

Fig. 2 HTS Z’ value and inhibition frequency. a Diagram of number of 384 wells plates grouped by the same Z’ value. In total, the Z’ value was calculated on 378 plates. b Inhibition percentage frequency distribution of the 119,059 compounds tested. The dotted line represents the 41% inhibition cut off limit.
The screening assay 28 luciferase reporter can represent a major confounding factor in those with the NF54 pfs16 gene.

PyLUC (Photinus pyralis) click beetle CBG99 luciferase signal and to confirm the activity on gametocytes from two NF54 strain derived lines: pfs16-GFP-PyLUC line (top left and middle) whereas 331 (52%) resulted in the NF54 hsp86-PpyRE13 strain. Cross red lines, settled at 40% inhibition for each axis, divides the plot in four fields.

As presented in Fig. 3b, 185 compounds (29%) were confirmed active in both lines (top right field), whereas 331 (52%) resulted poorly active in both lines (bottom left field). Assays with gametocytes of the NF54 hsp86-PpyRE13 line generally yielded more confirmed compounds (right top and bottom fields) than those with the NF54 pfs16-GFP-PyLUC line (top left and right fields). This strongly suggests that the inhibition of the luciferase reporter can represent a major confounding factor in the screening assay 28-31. In fact, the NF54 pfs16-GFP-PyLUC line shares same pfs16 promoter with the screening strain but differs in the reporter luciferase.

Altogether, this work suggested that differences between the line used in the HTS and those used in the confirmation assays (e.g., the different luciferase promoters, the different luciferase enzymatic properties, such as pH dependence and thermal stability and other unrelated factors) may contribute to differential compound activities and confirmed the value of early adoption of confirmation assays to make sure to select valuable compounds only.

In order to avoid advancing too many similar compounds, we performed a clustering based on the Taylor Butina algorithm 32, a non-hierarchical clustering method that ensures that each cluster contains molecules with a certain cut-off (or threshold) distance from a central compound. Circular fingerprints with radius 2 and 2048 bits were generated using the RDKit software 33 with the purpose of generating a similarity matrix based on a Tanimoto index 34. The effective number of neighbours for each molecule was calculated based on the Tanimoto level (0.8) used for clustering. This procedure gave a collection of 371 clusters, 205 of which were singletons. Subsequently, the selected set was subjected to quality control by LC-MS to check compound identity and purity (acceptable purity criteria set to be >90% peak area in the diode array trace). Altogether, the activity data on the three parasite lines, the compound clustering, the compound QC and medicinal chemist evaluation led to the identification of 84 molecules (Supplementary Data 2) that were progressed through the validation funnel. Among these, besides compounds that were active in all three gametocyte assay systems, we also included 15 compounds with interesting chemical properties that were active in the NF54 hsp86-PpyRE13 and 3D7elo1-pfs16-CBG99 strains only as we speculated that, being both the promoter and the reporter different between the two lines, they were likely true positives.

**Fig. 3 Hit confirmation and selectivity analysis.** The number of replicates for each experimental point was one for all the assays due to the technical challenges related to gametocyte assays. a Distribution chart representing the percentage of inhibition of 960 compounds in HeLa cells proliferation assay at 10 µM and in 3D7elo1-pfs16-CBG99 gametocyte assay at 20, 4, and 0.8 µM. The light blue and violet areas represent the selected and the discharged compounds, respectively. Dot horizontal line inside the areas indicates the median inhibition percentage (thick line) and top and bottom quartiles (thin lines). b Scatter plot of the percentage inhibition of PLuc assays of 638 compounds on NF54 pfs16-GFP-PyLUC strain vs NF54 hsp86-PpyRE13 strain. Cross red lines, settled at 40% inhibition for each axis, divides the plot in four fields. c Bubble scatter plot (log-log) of the selected 84 compounds potency (nM) on asexual stages (pLDH assay) vs gametocytes (PLuc assay) in the NF54 hsp86-PpyRE13 strain. The bubble colours correspond to the degree of potency of the HeLa proliferation assay coded by the column on the right.
Selectivity profiling. With the selected 84 compounds, the subsequent step aimed at determining the gametocyte inhibition potency and the parasite stage selectivity between the gametocytes and the asexual stages in the NF54 hsp86-PpyRE13. IC_{50} determinations showed that most compounds (64) were active against parasites in the micromolar range (≥1 µM) while 21 were sub-micromolar compounds. In terms of selectivity, 53 compounds (62%) had comparable potencies on gametocytes and on asexual stages, whereas 32 compounds (38%) had gametocyte selectivity values ranging from 2- to 268-fold (Fig. 3c). Similar data were obtained considering the gametocyte IC_{50} values measured in the NF54 pfs16-GFP-PyLUC strain (see Supplementary Data 2).

In addition, we tested the 84 compounds in a dose-response fashion on HeLa cells to quantitatively monitor their cytotoxicity potential. This assay revealed that most compounds (51, 60%) were more active (greater than two-fold) on gametocytes than in HeLa, irrespectively to the anti-axial stage or the anti-gametocyte activity (colour scale in Fig. 3c).

To further prioritize the 84 compounds, those with and IC_{50} below 3 µM in the NF54 pfs16-GFP-PyLUC gametocyte assay were selected. This set was further grouped in 14 groups and 29 singletons based on their chemical structure. Among these compounds, the elimination of those with a gametocyte/host selectivity less than five-fold and those with poor lead-like properties/tractability led to a final set of 30 molecules that were progressed to the male gamete eflagellation assay.

Activity of selected compounds in mosquito parasite stage assays. The above selected 30 compounds were subjected to a phenotypic male gamete eflagellation assay. This assay measures the viability of male gametocytes by quantitating the final step of maturation into motile microgametes in vitro. This process naturally occurs in the mosquito gut within minutes from the blood meal. The acquisition of time lapse videos of gamete motility in bright field microscopy and the subsequent analysis to count eflagellation centres were performed in an automated HTS format in 384-well plates. The compounds were tested in two independent biological replicates at the concentration of 1 µM. Methylene blue and compound DDD01035881 (compound 84) were used as positive controls. The results of this study led to the identification of 16 compounds with anti-eflagellation activity (Fig. 4). Among those showing an inhibition of the male gamete eflagellation greater than 50%, nine were further progressed for testing their transmission blocking activity (Fig. 5a). The standard membrane feeding assay (SMFA) is used to measure the parasite transmission through mosquito. In this assay, mature stage V gametocytes of the P. falciparum strain NF54-hsp70-luc were treated with 1 and 10 µM of the nine selected compounds for 24 h, added with human red blood cells and serum to achieve a haematocrit of 50% and then fed to mosquitoes. Luminescence was analyzed in the mosquito salivary glands 8 days post-feeding. Compound MMV0048 was used as positive control and, as expected, it abolished the parasite transmission at both concentrations. With the exception of compound 64, all compounds showed a high level of transmission inhibition at 10 µM. At the concentration of 1 µM, compound 53 was the most active, with about 100% inhibition, followed by compounds 13, 16, and 39 (Fig. 5b).

In summary, the screening of approximately 120,000 compounds against 3D7e01-pfs16-CBG99 gametocytes led to the identification of 960 compounds with a hit rate of about 0.8%. Counter-screenings against HeLa cells and two alternative gametocyte systems (NF54 hsp86-PpyRE13 and NF54 pfs16-GFP-PyLUC), together with hit quality control and a chemical clustering analysis, restricted the set of hits to 84 molecules. The evaluation of the gametocyte vs asexual stage selectivity led to the selection of 30 compounds that were assessed in a male gamete eflagellation assay. Active molecules in this assay led to the final selection of nine compounds that were tested in the standard membrane-feeding...
In this work, we developed a screening funnel (Fig. 6) for *P. falciparum* transmission blocking drug discovery starting with a simple, sensitive, robust and high-throughput assay followed by a second 41 % inhibition cut-off screen selecting 960 compounds (hit rate of about 0.8%). Three counter screening assays were performed on these compounds at fixed doses, a HeLa cell proliferation assay and two PfLuc assays on gametocytes of the NF54 hsp86-PpyRE13 and of the NF54 pfs16-GFP-PyLUC strains. An additional quality control assay and a chemical clustering analysis restricted the number of compounds to 84. These were filtered based upon potency as determined by full dose response assays complemented with a pLDH asexual stage assay on the NF54 hsp86-PpyRE13 strain. Results led to select 30 compounds to be tested in a male gamete exflagellation assay, whose results led to the final selection of 9 compounds tested in the standard membrane-feeding assay (SMFA), of which 8 turned out to be able to possess transmission blocking activity. Three of the latter represent, to our knowledge, novel chemotypes of *P. falciparum* transmission blocking molecules.

Discussion

Ten years ago two milestone HTS campaigns populated the antimalarial drug discovery pipeline of hits against asexual blood stages\(^1\), establishing a notable portion of the current antimalarial drug discovery portfolio. The size and diversity of the chemical space interrogated for asexual parasite killing activity undoubtedly represented a key factor in the success of that endeavour. These and other HTS campaigns contributed to the creation of the “Malaria Box”, a set of 400 promising antimalarial leads and tools most of which, however, resulted to be poorly or at all active on mature gametocytes\(^19,36\).

So far, compounds with anti-axial stage activity are, in most cases, unable to kill other stages of parasite development, including gametocytes and liver stages. It has been speculated that the biology of the proliferating asexual parasites is somewhat different from that of the non-dividing, terminally differentiated gametocytes. This is thought to be particularly true for mature stage V gametocytes. On the other hand, the results of gametocyte HTS\(^13,36\) led to the identification of both gametocyte-specific and dual active compounds, with the latter being quite frequent. This suggests that large anti-gametocyte HTS are likely to also discover novel anti-axial stage compounds.

In this work, we developed a screening funnel (Fig. 6) for *P. falciparum* transmission blocking drug discovery starting with a simple, sensitive, robust and high-throughput assay followed by a set of counter-screening and validation assays to test the gametocyte specific activity of the identified molecules. We screened almost 120,000 compounds, more than one third of the number globally interrogated so far in gametocyte HTS of diverse compound libraries\(^7-13,15-17\).

The amplitude of the primary screening was made possible by the adoption of a luciferase-based *P. falciparum* gametocyte assay where the CBG99 luciferase reporter expression was driven by the sexual stage specific *pfs16* promoter. The screening protocol is completely homogeneous and uses non-purified gametocyte culture. To develop the signal, the simple addition of D-luciferine, with no added ATP, was required before luminescence measurement.

The validation of the 638 hits, confirmed on the primary assay, was performed using gametocyte assays relying on two structurally different luciferases driven by two different parasite promoters. The results revealed that a rather big portion of the hits was likely ascribed to a direct inhibition of the click beetle CBG99 luciferase enzyme. Differently from other screening funnels, relying on more complex and lower throughput assays, interrogating different gametocyte processes\(^14,37\), the early and higher throughput use of these parasites lines conveniently and efficiently identified molecules to be directly progressed to more predictive phenotypic assays like male gamete exflagellation and SFMA.

In addition, this approach allowed for the early investigation of mature gametocyte vs asexual stage selectivity. We observed that 53 compounds (62%) had comparable potencies on gametocytes and on asexual stages, whereas 32 compounds (38%) had gametocyte selectivity values ranging from 2- to 268-fold. These results show that a HTS using gametocyte viability as primary readout can produce both dual active and gametocyte specific hits, suggesting that the parasite biology at least in part overlaps among different lifecycle stages.

In this work, to further prioritize and validate hit molecules, we used the male gametocyte viability assay that is universally adopted for its high predictivity of parasite infectiousness to mosquito. Male gametocytes are a minor fraction of the parasite.
sexual stages, given the female biased sex ratio of Plasmodium gametocytes. In the NF54 genetic background used here, the typical male to female ratio is 1 to 10. Nevertheless, the efficient inhibition of male gametocytes is highly desirable for parasite transmission blocking drugs as these sexual stages appear to be critical in infections with very low gametocytaemias, a common feature in the asymptomatic carriers, who are ideal recipients of transmission blocking drugs. This choice may have led us to miss compounds active only on female gametocytes. However, we decided not to introduce a female gametocyte specific assay as the upstream luciferase gametocyte assays contained, according to the previously mentioned sexual bias, mainly female gametocytes. Hence, compounds that failed to inhibit exflagellation may be reconsidered in the future as possible female specific compounds.

The final SFMA validation allowed us to identify seven chemotypes able to block malaria parasite transmission through mosquitoes via their inhibitory activity on P. falciparum gametocyte viability. Four of these have been previously identified in SMFA screenings based on orthogonal unrelated assays. A similar structure was identified in a HTS on asexual stages and the chemically related hits 48, 52, and 56, reduced male gamete exflagellation to 20–30% that is considered as an acceptable starting point for not optimized compounds. In SMFA, compound 53 inhibited parasite transmission after a 24 h treatment at 1 μM was equivalent to that of the positive control MMV0048.

Compound 39, first identified in a HTS on asexual stages as TCMDC-125769, was subsequently shown active on gametocytes in a parasite ATP assay. Importantly, our work adds that this compound efficiently blocks parasite transmission in mosquito.

Compound 31, a moderate transmission blocker hit, is a singleton. A similar structure was identified in a HTS on 110,000 drug-like compounds as an inhibitor of P. falciparum haem detoxification protein HDP involved in hemozoin formation (US20070148185).

The 2,4-diamino-pyrimidine chemotype of compounds 63, 64, and 65 was identified on asexual stages and modifications were introduced to study its proposed kinase inhibitory activity. Our work extended the functional characterization of these compounds to demonstrate activity in blocking parasite transmission.

Importantly, this work also identified, to the best of our knowledge, three novel chemotypes, which ranked amongst the most active compounds tested here in blocking P. falciparum transmission through mosquito.

Compound 13 is the second most active compound in SMFA. The chemotype of this singleton has not been previously described in any HTS on malaria parasites. The symmetrical structure in a double 4-piperidinyl benzyl alcohol linked by a short alkyl chain generated an appealing hit with a potential for further optimization. In silico calculation of its physicochemical properties (logD 2.2 and TPSA 47, MW 491.5, 4 HBD, 2 HBA) demonstrated that there is still room for additional exploration without impacting the Lipinski’s Rule of Five.

Compound 16, the third most active hit in SMFA with a gametocyte specificity of around 6-fold, is a singleton characterized by an imidazo[1,2-a]pyridine core disubstituted in position 2 and 3. It is unknown if the charged N-oxide pyridine is crucial for the potency or if other cores can be tolerated in the central region. Even if the aromatic thiol might be vital for the geometry of the linker, it is not ruled out that ethers or amines might be allowed. Although this structure has not been identified as an anti-gametocyte hit, interestingly, other imidazopyridines, with a different pattern of substitutions, have been shown to inhibit P. falciparum gametocyte viability across several orthologous assays.

Compound 69 is another chemotype that was not previously published as an antimalarial hit with a gametocyte specificity of around six-fold. The furanyl-piperidinyl scaffold is not present in any reported transmission blocking drug discovery study. A comparable substituted moiety with distinct decoration on the piperidinyl core is reported in patent number WO2015006752A1 as a p38 MAP Kinase inhibitor VI in combination with other antimalarial drugs. Still, compound 69 looked both small and flexible to be further explored and ultimately optimized.

Prior to our work, a cumulative set of about 300,000 compounds was screened for its activity on P. falciparum gametocytes in a variety of assays, making the chemical space explored by gametocyte HTS approximately ten percent of that tested on asexual stages. The present work shows that it is possible to notably extend the amplitude of anti-gametocyte hit identification campaigns and that our screening funnel was further validated by the identification of chemical series that were selected by previous screenings based on orthogonal unrelated assays.

We believe that gametocyte HTS will drive the identification of novel and diverse transmission blocking chemotypes suitable for drug discovery but also chemical probe to interrogate the parasite biology. Furthermore, we not only identified here multiple dual active hits with similar potency against gametocytes and asexual stages, but also molecules preferentially active on gametocytes with the potential to be developed into drugs able to clear residual gametocytes after treatment of malaria episodes or for mass drug administration in asymptomatic populations to hit local transmission hotspots. While future mechanistic studies are warranted for the elucidation of the underlying biology, the availability of novel starting points is extremely relevant for the identification of new drugs to ultimately contribute to malaria elimination and the eradication of parasite.

Methods

**Compound collection.** CNCCS represents a public-private consortium (www.cnccs.it) whose objective is the construction of a collection of compound molecules. In addition to FDA and/or EMA approved drugs, the collection contains a range of chemotypes, from both commercial and non-commercial suppliers, with an optimized structurally diversity (average Tanimoto distance from the nearest neighbour of 0.38; and an average molecular weight of 370 Daltons. The size of the library comprises approximately 120,000 small molecules not biased toward any particular target nor diseases oriented. While the collection was optimized for structural diversity, it maintains an attractive distribution of physicochemical properties (e.g., calculated logD, sp3 character, hydrogen bond donor/acceptors and total polar surface area).

**Compound similarity search.** After hit confirmation, compound similarity searches were performed by generation of circular Morgan fingerprints (radius 2, 2018 bits) for the test compounds using open source RDKit software (http://www.rdkit.org/ release 2014_09_2). The molecular representations generated were used to perform ligand based virtual screening against the target database (i.e., our own screening collection) that is described above or against a subset of the public ZINC database (http://www.zinc.docking.org). Similarity was assessed by the Tanimoto index between the reference and target structures using a cut-off (or threshold) of 0.6. Similar compounds were clustered using Taylor-Butina clustering; a non-hierarchical clustering method that ensures that each cluster contains molecules with a set cut-off distance from the central compound. Compounds selected for purchase or screening follow up were chosen from the most populated clusters, with either the central compound or closed analogues (based on visual inspection) being used to represent the compound cluster. All selected compounds were quality controlled by UPLC-MS prior to testing. The identity and purity of compounds that were not previously associated to antimalarial activity (compound 96, 13, and 16) was verified (Supplementary Fig. 2).

**Parasite lines and culture protocols.** P. falciparum asexual parasites of the lines and clones indicated below were cultured in type A/0+ human erythrocytes at 5%
hematoctr and 0.5–10% parasitemia in RPMI 1640 (Life Technologies). The medium was supplemented with 10% heat inactivated O- human serum (IBBI, Memphis, USA), 50 mM N-acetyl glucosamine (4% CO2, 3% O2, and 93% N2) by established methods (Trager and Jensen 1976). Gametocyte production was induced by seeding asexual parasite cultures at 0.1% parasitaemia and 5% hematoctrit with no further addition of uninfected red blood cells. At the appearance of the stage 4 gametocytes at day 4, 50 mM N-Acetyl glucosamine (NAG) was added for 3 days to clear residual asexual parasites, followed by additional 5 days of culturing in absence of NAG. Parasite lines used in this work were the reference wild type clone 3D7A45, line 3D7, NF54-PsyRE13, and line NF54- PsyRE13 produced for this work as described below.

Production of the P. falciparum line NF54-psyRE13. The PsyRE13 luciferase mutant gene from the firefly Photinus pyralis was kindly provided by Dr. B. Branchini, Connecticut College, London CT, USA (manuscript in preparation). The multistep cloning strategy to obtain the pCR2.1-attP-psyRE13 plasmid, carrying the PsyRE13 red luciferase under the P. falciparum hsp86 constitutive promoter, was as follows (Supplementary Fig. 1). In brief, the psyRE13 plasmid was digested with Spel and Ncol restriction endonucleases (New England Biolabs) and cloned into a Spel-Ncol-digested psyRE1-given 3-naplam to obtain the intermediate psyRE1-given 3-naplam plasmid. Then the psyRE1-given 3nplasmid was Xhol-Notl digested to clone the given 3n-psyRE13 cassette into the Xhol-Notl-digested pCR2.1-attP-CBG997 plasmid to finally obtain the pCR2.1-attP-psyRE1-given 3nplasmid. Parasites from the P. falciparum NF54-psyRE13 line, containing a Bbx1 attB site in the g6 gene, were grown to the 6% ring parasitaemia and transfected with both 100 µg of pCR2.1-attP-psyRE1-given 3nplasmid and 100 µg of plINT-psyRE1 plasmids, to obtain the NF54-psyRE1-given 3nplasmid. Transfection was via electroporation using a BioRad electroporator with 0.31 kV voltage, 960 µF capacitance, and 2.5 nM WR99210 and 3 days later they were allowed to recover in drug-free medium. To confirm the integration of the pCR2.1-attP-psyRE1-given 3nplasmid construct into the genome of the NF54-psyRE1-given 3n plasmid analyses were performed using Cg6-P1-Plasm_P2 primers and Plasm_P4_Cg6_P3 primers (Supplementary Table 1).

P. falciparum gametocyte luminescence assay. Parasite gametocyte luminescence assays were performed in 384-well plates (Thermo, 4332, USA) by infecting red blood cells seeded at 40 µL/well at a haematocrit of 0.625% for the strain 3D74-psyRE1-CBG997. 1.875% for both strains NF54-psyRE1-PsyRE13 and NF54-psyRE1-GFP-psyRE1. Assay plates were performed in the medium: 10.44 g/L RPMI 1640 Medium (Gibco, 51800043), 5.95 g/L HEPES (Sigma, H4034), 50 mg/L Hypoxanthine (A) and acetonitrile containing 0.1% formic acid (B) with a ow rate 0.5 mL/min. Data analysis was carried out with GraphPad 9.0.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Source data for figures are provided in Supplementary Data 1.

References

1. WHO. World Malaria Report 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021 (2021).
2. WHO. Global technical strategy for malaria 2016–2030. https://apps.who.int/iris/bitstream/handle/10565/200334/9789241565318-eng.pdf;jsessionid=440A2E86B5B0F9364EB7D02B39DCC4A4?sequence=1 (2016).
3. Burrows, J. N. et al. New developments in anti-malarial target candidate and product profiles. Malar. J. 16, 1–29 (2017).
12. Tanaka, T. Q. et al. Potent Plasmodium falciparum gametocytocidal activity of
11. Sun, W. et al. Chemical signatures and new drug targets for gametocytocidal
9. Tanaka, T. Q. et al. A quantitative high throughput assay for identifying
gametocytocidal compounds. Mol. Biochem. Parasitol. 188, 20–25 (2015).
10. Spicer, T. P. et al. Identification of antimalarial inhibitors using late-stage
gametocytes in a phenomenotypic live/dead assay. SLAS Discov. 24, 38–46 (2019).
13. Delves, M. J. et al. A high throughput screen for next-generation leads
targeting malaria parasite transmission. Nat. Commun. 9, 1–11 (2018).
14. Reader, J. et al. Multistage and transmission-blocking targeted antimalarials
discovered from the open-source MMV Pandemic Response Box. Nat. Commun. 12, 269 (2021).
15. Sun, W. et al. Novel lead structures with both Plasmodium falciparum
gametocytocidal and sexual blood stage activity identified from high
throughput compound screening. Malar. J. 16, 1–11 (2017).
16. Luconatoni, L., Fidock, D. A. & Avery, V. M. Luciferase-based, high-throughput
assay for screening and profiling transmission-blocking compounds against
plasmodium falciparum Gametocytes. Antimicrob. Agents Chemother. 60, 2097–2107 (2016).
17. Sanders, N. G., Sullivan, D. J., Mlambo, G., Dimopoulos, G. & Tripathi, A. K. Plasmodium
gametocyte screen identifies novel chemical classes with Plasmodium
falciparum transmission blocking activity. PLoS One 9, 1–13 (2014).
18. Fidock, D. A. Priming the antimalarial pipeline. Nature 465, 297–298 (2010).
19. Luconatoni, L. et al. A simple and predictive phenotypic High Content Imaging
assay for Plasmodium falciparum mature gametocytes to identify malaria
transmission blocking compounds. Sci. Rep. 5, 1–14 (2015).
20. Cevenini, L. et al. Multicolor bioluminescence boosts malaria research:
Quantitative dual-color assay and single-cell imaging in Plasmodium
falciparum parasites. Anal. Chem. 86, 8814–8821 (2014).
21. D’Alessandro, S. et al. A Plasmodium falciparum screening assay for anti-
gametocyte drugs based on parasite lactate dehydrogenase detection. J. Antimicrob. Chemother. 68, 2048–2058 (2013).
22. Leillière, J. et al. Activity of clinically relevant antimalarial drugs on
Plasmodium falciparum mature gametocytes in an ATP bioluminescence
‘transmission blocking’ assay. PLoS One 7, e53019 (2012).
23. Tanaka, T. Q. & Williamson, K. C. A malaria gametocyte assay using
oxidoreduction indicator, alamarBlue. Mol. Biochem. Parasitol. 177, 160–163
(2011).
24. Ruecker, A. et al. A male and female gametocyte functional viability assay
to identify biologically relevant malaria transmission-blocking drugs.
Antimicrob. Agents Chemother. 58, 7292–7304 (2014).
25. D’Alessandro, S. et al. A chemical susceptibility profile of the Plasmodium
falciparum transmission stages by complementary cell-based gametocyte
assays. J. Antimicrob. Chemother. 71, 1148–1158 (2016).
26. Zhang, J.-H., Chung, T. D. Y. & Oldenburg, K. R. A simple statistical
parameter for use in evaluation and validation of high throughput screening
assays. J. Biomol. Screen. 4, 67–73 (1999).
27. van der Watt, M. E. et al. Potent Plasmodium falciparum gametocytocidal compounds identified by exploring the kinase inhibitor
chemical space for dual active antimalarials. J. Antimicrob. Chemother. 73, 1279–1290 (2018).
28. Heitman, L. H. et al. False positives in a reporter gene assay: Identification and
synthesis of substituted N-pyridin-2-ylbenzamides as competitive inhibitors of
firefly luciferase. J. Med. Chem. 51, 4724–4729 (2008).
29. Thorne, N., Inglese, J. & Auld, D. S. Illuminating insights into firefly luciferase and
other bioluminescent reporters used in chemical biology. Chem. Biol. 17, 646–657 (2010).
30. Borrell, A. et al. InterPred: a webtool to predict chemical autofluorescence and
luminescence. Nucleic Acids Res. 48, W586–W590 (2020).
31. Yang, Z. Y. et al. Structural analysis and identification of false positive hits in
Luciferase-based assays. J. Chem. Inf. Model. 60, 2031–2043 (2020).
32. Butina, D. Unsupervised data base clustering based on daylights fingerprint
and Tanimoto similarity: A fast and automated way to cluster small and large
data sets. J. Chem. Inf. Comput. Sci. 39, 747–750 (1999).
33. Riniker, S. & Landrum, G. A. Open-source platform to benchmark
fingerprints for ligand-based virtual screening. J. Cheminform. 5, 26 (2013).
34. Rogers, D. J. & Tanimoto, T. T. A computer program for classifying plants.
Science 132, 1115–1118 (1960).
35. Paquot, T. et al. Antimalarial efficacy of MMV390048, an inhibitor of
Plasmodium phosphatidylinositol 4-kinase. Sci. Transl. Med. 9, eaaz9735 (2017).
36. Sun, W. et al. Chemical signatures and new drug targets for gametocytocidal
drug development. Sci. Rep. 4, 37443 (2015).
37. Birkholtz, L. M., Coetzer, T. L., Mancama, D., Leroy, D. & Alano, P.
Discovering new transmission-blocking antimalarial compounds: Challenges
and opportunities. Trends Parasitol. 32, 669–681 (2016).
38. Bradley, J. et al. Predicting the likelihood and intensity of mosquito infection
from sex specific plasmodium falciparum gametocyte density. Elife 7, 1–13
(2018).
39. Ouedraogo, A. L. et al. Dynamics of the human infectious reservoir for malaria
determined by mosquito feeding assays and ultrasensitive malaria diagnosis in
Burkina Faso. J. Infect. Dis. 213, 90–99 (2016).
40. Spangenber, T. et al. The open access malaria box: A drug discovery catalyst
for neglected diseases. PLoS One 8, e62906 (2013).
41. Vallone, A. et al. Antimalarial agents against both sexual and asexual parasite
growth: structure-activity relationships and biological studies of the Malaria
Box compound 1-[5-(4-bromo-2-chlorophenyl)furanyl-2-yl]-N-[piperidin-4-yl]methyl[methanamine (MMV019918) and analog. Eur. J. Med. Chem. 150, 698–718 (2018).
42. Gamo, F. J. et al. Thousands of chemical starting points for antimalarial lead
identification. Nature 465, 305–310 (2010).
43. Phuangwai, O. et al. Everteaswari, A. et al. Antimalarial activity and cytotoxicity
of 2,4-diamino-pyrimidine-based kinase inhibitors. Eur. J. Med. Chem. 124, 896–905 (2016).
44. Gupta, S. K., Schulman, S. & Vanderberg, J. P. Stage-dependent toxicity of
N-acetyl-glucosamine to Plasmodium falciparum. J. Protozool. 32, 91–95
(1985).
45. Walliker, D. et al. Genetic analysis of the human malaria parasite Plasmodium
falciparum. Science 236, 1661–1666 (1987).
46. Nkrumah, L. J. et al. Efficient site-specific integration in Plasmodium
falciparum chromosomes mediated by mycobacteriophage Bbx1 integrase.
Nat. Methods 3, 615–621 (2006).
47. Siciliano, G. et al. A high susceptibility to rebox imbalance of the transmissible
stages of Plasmodium falciparum revealed with a luciferase-based mature
gametocyte assay. Mol. Microbiol. 104, 306–317 (2018).
48. Vos, M. W. et al. A semi-automated luminescence based standard membrane
feeding assay identifies novel small molecules that inhibit transmission of
malaria parasites by mosquitoes. Sci. Rep. 5, 1–13 (2015).

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Author contributions
A.B., P.A., and G.P. conceived the study, interpreted the results and wrote the manuscript with input from all authors. C.A. collected and analyzed data. G.S. performed and analyzed gametocyte and exflagellation assays and produced the hsp86PR1E13 construct. R.G., C.L., O.C., and M.B. performed parasite and HeLa cell assays. A.S. and A.P. did QC for the data analysis. A.L. contributed to the manuscript, C.T. reviewed and approved the manuscript. All authors reviewed and approved the manuscript.

Competing interests
The authors declare no competing interests.
