

Discovering new transmission-blocking anti-malarial compounds: challenges and opportunities

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Abstract

The ability to target human-mosquito parasite transmission challenges global malaria elimination. However, it is not obvious what a transmission-blocking drug will look like; should it 1) target only parasite transmission stages; 2) be combined with a partner drug killing the pathogenic asexual stages or 3) kill both the sexual and asexual blood stages, preferably displaying polypharmacology. The development of transmission-blocking anti-malarials requires objective analyses of the current strategies. Here, pertinent issues and unanswered questions regarding the target candidate profile of a

transmission-blocking compound, and its role in malaria elimination strategies are highlighted and novel perspectives proposed. The essential role of a test cascade that integrates screening and validation strategies to identify next generation transmission-blocking anti-malarials is emphasised.

Trends

Global efforts to eliminate and/or eradicate malaria are targeting all stages of parasite development. Disrupting the transmission of parasites from humans to mosquitoes is an attractive target that is amenable to chemotherapeutic intervention.

Screening compounds that kill gametocytes is performed against a poorly understood biological background of the target cells.

A smart screening strategy that introduces a range of biologically informative and clinically important assays early in the pipeline maximises the confidence in the identification of lead compounds.

Knowledge gaps and questions regarding the identification, characterisation, and optimal profile of transmission-blocking drugs are highlighted and novel perspectives proposed.

Keywords: malaria, gametocytes, transmission, drug discovery, elimination

Malaria: from control to elimination

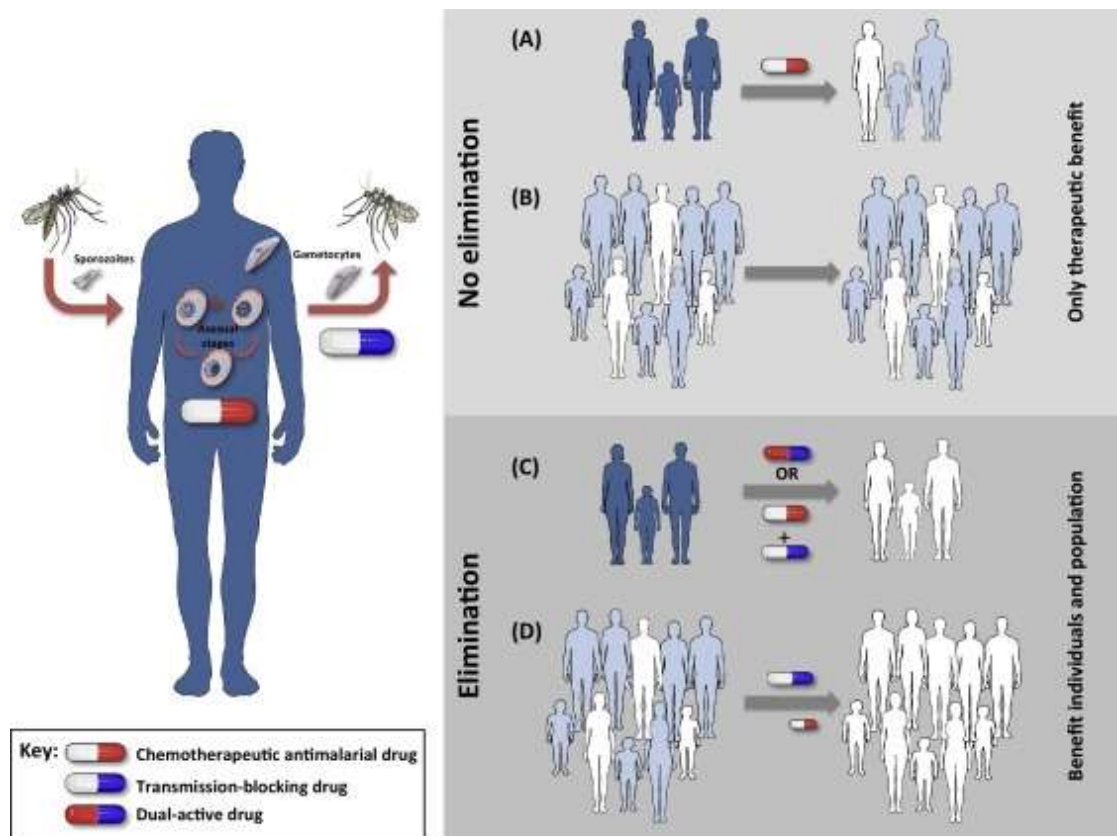
The unsustainable cost of controlling malaria has led to concerted global efforts to **eliminate** (see Glossary) and eventually **eradicate** the disease, resulting in a 37% global decline in malaria incidence since 2000. However, despite this tremendous success, 3.2 billion people remain at risk and in 2015 an estimated 214 million cases of malaria resulted in 438 000 deaths [1]. One of the main contributing factors is the sustained transmission of the causative *Plasmodium* parasites between humans through *Anopheles* mosquito vectors. The ability to block transmission relies on identifying (and treating) asymptomatic or semi-immune human hosts carrying transmissible forms of the parasite and representing major reservoirs of continued infection [2]; eliminating mosquito vectors through multiple and integrated strategies [3]; and eliminating the parasite pool in malaria patients, which, in the absence of a vaccine, still relies solely on chemotherapy and prophylaxis to prevent new or re-infection [4]. The latter is under threat due to resistance against current anti-malarial drugs.

Parasite **population bottlenecks** appear in transition phases of the *Plasmodium* life cycle during which parasites are transmitted between the vector and human hosts [5]. Prevention of **sporozoite** transmission from the mosquito has thus far relied entirely on vector control and on measures to prevent mosquito bites [5]. On the other hand, the transmission of **gametocytes** (the intra-erythrocytic sexual stages) to the mosquito is potentially more amenable to direct intervention, as it is easily targetable within the human blood compartment.

However, targeting gametocytes remains challenging since vast gaps exist in our knowledge of the biology of the sexual stages of *Plasmodium*. Gametocytes are highly specialized cells, very different to the asexual pathogenic stages [6]. In *P. vivax*, gametocytes require only a slightly longer time to develop than that required for asexual stages to complete their multiplication cycle. By contrast, gametocytes of *P. falciparum* reach maturity in a uniquely long period of 10-12 days compared to the 48 h asexual cycle. In this species major morphological differences distinguish the asexual parasites

from gametocytes, from whose elongated shape *P. falciparum* derives its name. Five distinct morphological and biochemical stages of gametocyte development (stages I-V) are conventionally identified [7], with immature stage I-IV gametocytes sequestering in tissues (e.g. bone marrow) during development and only mature stage V gametocytes circulating in the blood stream where they can be transmitted to mosquitoes [8]. After the stochastic, epigenetically driven [9-12] gametocyte conversion rate of ~1% of the asexual population [6], the immature stage I-III gametocytes are to some extent biochemically more aligned to asexual parasites than their stage V partners [8] whose metabolism effectively decreases to only retain household activities such as ATP production and redox maintenance [13]. This raises unique issues in the identification of drugs active on *P. falciparum* gametocytes, and specifically on the mature stage V forms, compared for instance to those of *P. vivax*, in which drugs against the asexual stages are much more often also active against gametocytes. This paper will consequently focus on strategies to target the transmission stages in *P. falciparum*.

Currently, only artesunate, artemether, methylene blue and primaquine are active against gametocytes, with primaquine the only approved gametocidal drug. The use of these compounds is threatened by emerging resistance to artemisinin derivatives and toxicity concerns in the case of primaquine, which causes haemolysis in glucose-6-phosphate dehydrogenase deficient individuals [14]. This paper will argue that the discovery of new transmission-blocking anti-malarials should be strategically driven by how such compounds will ultimately be deployed (Figure 1, Key figure). Pertinent discussion points and knowledge gaps in the identification, characterisation and classification of a transmission-blocking drug will be highlighted. Novel perspectives and expert opinions are proposed to guide the way forward towards achieving the goal of identifying transmission-blocking anti-malarials.



Trends in Parasitology

Figure 1.

Key Figure: Strategies towards Malaria Elimination: The Debate.

Malaria control and elimination through parasite targeting is currently entirely dependent on the use of chemical interventions. The development of new transmission-blocking drugs may result in a drug targeting only gametocytes or it may have dual activity against gametocytes and asexual stages. To treat symptomatic patients (blue), chemotherapeutics are used to kill pathogenic asexual forms of the parasite, but transmissible sexual gametocytes may not be eliminated (A). Asymptomatic, semi-immune carriers of gametocytes (pale blue) remain undetected and untreated in populations and perpetuate parasite transmission to the mosquito vector (B). In these scenarios, malaria will not be eliminated. To achieve elimination, the reservoir of gametocytes in the population has to be cleared. Thus, patient with malaria may be treated with either a dual-acting drug or a combination of a drug curing malaria plus a transmission-blocking drug (C). At the population level, administration of a transmission-blocking drug to asymptomatic gametocyte carriers will eliminate gametocytes, which will prevent transmission and thereby disrupt the parasite life cycle (D). However, this will only be achieved if these asymptomatic patients do not carry low-level asexual parasites, in which case they should be treated concomitantly with a chemotherapeutic drug.

Targeting transmission to eliminate malaria: what role will transmission-blocking anti-malarials need to fulfil?

Four challenges associated with anti-malarial drugs relevant to a malaria eradication agenda have previously been outlined: 1) blocking disease transmission by targeting sexual blood stage development in humans; 2)

elimination of liver-stages including preventing and/or eliminating relapse from hypnozoites; 3) developing chemical entities that overcome cross-resistance and 4) targeting vulnerable populations [5, 15, 16]. However, a provocative challenge to the above could be to prioritise blocking transmission as a primary objective, since this could provide an over-arching solution to achieve malaria elimination.

Of particular interest is the role that **transmission-blocking** compounds will need to fulfil (Figure 1). Dual-active compounds, i.e. single compounds that target both asexual blood stages and mature gametocytes equipotently, provide the possibility to consolidate several anti-malarial **target candidate profiles** (TCPs) into a single **target product profile** (TPP) (for full definition see Glossary)[5, 15, 16] but with increased risk of resistance development (Box 1). Combinations of more than one entity, one with asexual blood stage activity and one with transmission-blocking ability could decrease the risk of developing resistance, but are associated with increased development costs and pharmacological complexities. Lastly, the use of a 'transmission-blocking only' compound as a 'chemical vaccine' to prevent transmission holds promise and could be useful to eliminate the vast gametocyte reservoir in asymptomatic individuals within whole populations in a **mass drug administration** (MDA) scenario [17] or in targeted delivery to gametocyte carriers in a mass test and treat scenario, if carriers could be successfully identified. This will not directly benefit the asymptomatic gametocyte carrier but will 'indirectly' protect the community and greatly decrease the parasite pool. Such a strategy will always have to be used in parallel with the treatment of malaria patients, as well as those asymptomatic gametocyte carriers with low levels of asexual parasites. Safe transmission-blocking strategies could have a marked impact in high-transmission settings but could also be adapted to target only certain populations in transmission hotspots and be useful in (pre)-elimination settings. However, currently, the identification of transmission-blocking compounds is complex and faces numerous unique challenges as discussed below.

Box 1. Expanding the target candidate profile for transmission-blocking compounds

Target candidate profiles for transmission-blocking drugs in the context of human-to-mosquito screens are currently broadly defined as compounds with activity against mature gametocytes that translate to full blocking of mosquito infection in SMFAs (TCP3b). Practically, however, the design of a transmission-blocking screening cascade raises several questions, necessitating a clearer definition of strategies to address TCP3b.

Dual-activity: Current strategies advocate screening for compounds with activity against asexual parasites, with hits subsequently screened for transmission-blocking activity against mature gametocytes, thereby identifying 'equipotent' compounds with useful therapeutic and transmission-blocking activities (Figure 1). Such dual-active compounds may potentially be used as single-dose cures but with increased risk that resistance developed by asexual parasites could be conferred to gametocytes.

Sole gametocytocidal activity: Screening platforms could be aimed at *de novo* screening of compounds against gametocytes to identify transmission-blocking compounds. Hits should be tested against asexual parasites to confirm specific activity or to identify a dual-active compound. If a compound is only active against gametocytes (>10-fold selectivity [20]) it may be used to eliminate the reservoir of gametocytes in semi-immune asymptomatic carriers with a predictable 'reduced-risk' of inducing resistance. However, strategies that will allow efficacy, dosing and safety testing *in vivo* have not been clearly defined for transmission-blocking only compounds compared to those outlined for compounds active against asexual parasites and this could complicate their clinical development.

Combinations: Compounds active against asexual parasites and with a specific phenotype (e.g. TCP1 (fast parasite clearance) or TCP2 (long lasting molecules) could be combined with compounds with potent sole gametocytocidal activity (TCP3b) if PK/PD constraints could be addressed. This requires information such as stage-specificity and kinetics of action. Long-lasting asexual compounds combined with long-lasting gametocytocidal compounds may be very effective to target pathogenesis as well as transmission. TCP3b compounds able to target immature gametocytes will contribute to decrease the pool of transmissible mature gametocytes and may overcome the issue associated with some compounds whereby anti-asexual stage activity enhances the production of gametocytes.

Sex-specific targeting: Male gametogenesis appears to be more sensitive to drugs. Sex-specific compounds may be more likely to exclusively target the parasite and thus display a better cytotoxicity profile than those that target both sexes [27].

MOA: In all of the above scenarios, compounds could either 1) have the same MOA in asexual parasites, immature and mature gametocytes or 2) could target different mechanisms

in the various lifecycle forms of the parasite. In the latter case, this could potentially lead to pleiotropic compounds displaying polypharmacology with a reduced potential to develop drug resistance.

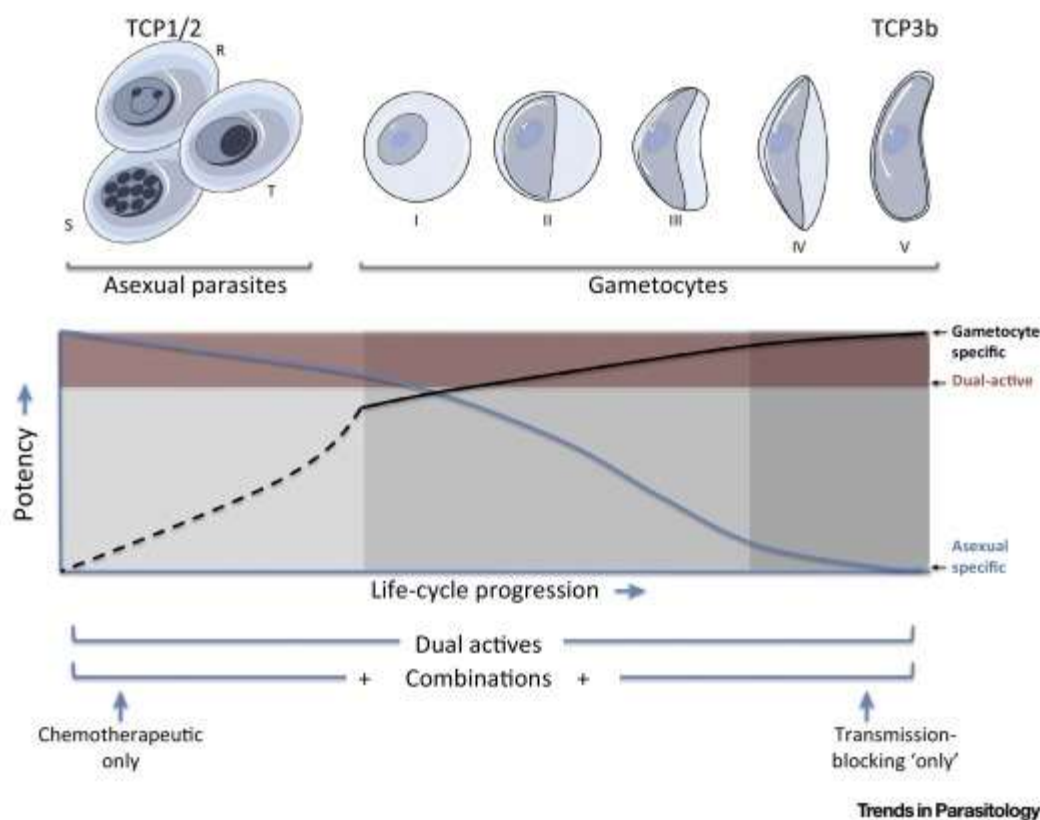


Figure I: Spectrum of activity of anti-malarial compounds and combinations. Screens against asexual parasites have identified a number of potent chemotherapeutic compounds (nM efficacy); however, typically these compounds maintain efficacy against early gametocyte stages but are not/less active against mature gametocytes (blue line). The converse is also true for gametocyte-specific compounds (black line) although correlation to asexual activity is not essential (dashed line). Dual-active compounds killing all stages may also be identified (red block) with 'equipotency' (i.e. <math><10</math>-fold difference in potency). Parasite images were adapted from freely available images from www.servier.com. R=ring, T=trophozoite and S=schizont asexual stages.

Transmission-blocking compounds: challenges to success

Phenotypic screening of more than seven million compounds for activity against the asexual blood stage pathogen has resulted in new chemical entities entering clinical development to ensure continued population of the drug development pipeline (www.mmv.org)[5, 18]. Similar approaches have been proposed to discover transmission-blocking compounds by screening against primarily mature gametocytes but this has been very difficult

compared to screening asexual blood stages, particularly because the apparent quiescence of mature gametocytes has hampered the development of cell-based screening assays specific to stage V gametocytes [19]. This limited metabolic repertoire restricts the 'druggable' pool of biochemical activities in these parasites [20].

Several points need to be addressed to devise strategies focussed on blocking human-to-mosquito transmission [5, 21]: 1) achieving bulk *in vitro* production of biological material (e.g. mature gametocytes from diverse parasite lines) that is viable, functional and amenable to downstream chemical interference assays; 2) developing robust technologies that interrogate the killing activity of chemical entities and developing high biological content assays to validate transmission-blocking activity; 3) increasing assay throughput; 4) identifying appropriate progression selection criteria; 5) using high biological content assays as early progression filters; 6) understanding pharmacological considerations of transmission-blocking entities including efficacy models and dosing; 7) addressing at an early step the issue of safety of these compounds. Although some of these aspects have been addressed, including gametocyte production and assay development (see e.g. [14, 19]), we are only starting to see applications of chemical library screening for gametocytocidal activity.

The Medicines for Malaria Venture (MMV) Malaria Box of 400 compounds (selectively active against asexual parasites [22]) has been screened worldwide for activity against stage IV/V gametocytes [20, 23-31]. Primary hit rates (>80% inhibition at 5 μ M) of 4.5-24% [20, 23, 27, 29] were achieved when screened against stage IV/V gametocytes; this increased to 33% against stage I-III gametocytes [23, 26]. Furthermore, although the rank-order of hits were similar, there was not complete overlap between MMV Malaria Box screens conducted with different assay platforms or between laboratories using the same assay platform [24, 25].

A number of groups have also screened more diverse and larger chemical libraries for gametocytocidal activity (Table 1). Similar to the Malaria Box results [23], the majority (>80-90%) of compounds targeting the asexual

parasite are ineffective against stage IV/V gametocytes [32] with little correlation between the anti-asexual and gametocytocidal activity [23, 33, 34]. Some chemical signatures for compounds targeting both asexual and gametocyte stages have been described [24] including endoperoxides, certain quinolines (including primaquine and mefloquine), anthracyclines, dihydroergotamine-type adrenergic agents and inhibitors of kinases, protein biosynthesis, the proteasome, protein modification / membrane trafficking and ion homeostasis [20, 32]. Additionally, certain chemical classes (e.g. diamidonaphthoquinones – DANQ - derivatives [32]) have dual activity but not against the same target, suggesting different asexual and sexual stage-specific **mode(s)-of-action (MOA)**. Such drugs showing **polypharmacology** additionally have the advantage of reducing the risk of resistance development [35].

De novo or naïve screening of large unbiased chemical libraries has yielded novel, gametocyte-specific chemical classes not previously reported to have anti-malarial activity [20, 29, 32, 33], supporting the notion that such strategies could yield transmission-blocking compounds. Certain scaffolds (e.g. targeting nucleic acid production, ATP production or fatty acid biosynthesis [20, 34]) have been shown to exhibit transmission-blocking activity by targeting gametogenesis, questioning the readout of mature gametocyte activity as the sole endpoint in transmission-blocking assays.

The data from library screens summarised in Table 1 illustrate the low gametocytocidal hit rates achieved and the necessity to explore vast numbers of compounds. This impacts on the rate of progress in identifying gametocytocidal compounds and highlights the key importance of designing and implementing a clear, rational and sustainable screening test cascade.

Smart screening of chemical libraries for transmission-blocking compounds

MMV proposed a comprehensive test cascade for TCP1 drugs (fast parasite clearance, reducing initial parasite load) and TCP2 drugs (longer duration partner drug to complete parasite clearance) targeting asexual parasite

development, however, a detailed strategy for TCP3 transmission-blocking-only chemical entities (drugs killing the non-dividing forms of the parasite) is still lacking [5]. A pragmatic approach to identify a limited set of leads or preclinical candidates has been advocated by both the MMV [5] and the Crimalddi Consortium [21], who have proposed the use of a two-step strategy where primary high-throughput screens against gametocytes serve as early strict filters to select hits that have to be tested and robustly validated with additional assays in secondary platforms designed to interrogate different biological activities. Compounds validated across several platforms all potentially provide chemical scaffolds for transmission-blocking profiling. The secondary screen test cascade should span the entire transmission pathway and include *in vitro* **orthogonal confirmatory assays** on gametocytes and, ideally, assays for at least **gametes** as filters prior to **standard membrane-feeding assays** (SMFAs).

Apart from designing the most appropriate cascade, several outstanding strategic questions remain regarding approaches to screen for gametocytocidal compounds, either *de novo* or dovetailing with asexual screens (Box 1). Transmission-blocking screening cascades have been developed by the Bill and Melinda Gates Foundation Gametocyte Project and the South African Malaria Transmission-blocking Consortium, with several points of overlap. These have been integrated in Figure 2, which represents an idealised screening strategy based on assays available within these Consortia that could be seen as one of the possible practical routes to discover and validate transmission-blocking compounds. Other appropriate assays based on individual laboratory resources may be used to achieve the same goal, which is ultimately to ensure that only compounds that have been comprehensively validated are selected for further development. A 3-tiered pipeline that follows a logical and structured flow of activities with clearly defined criteria for progression to the next stage is conceived to maximise relevant information and avoid duplication. Each tier is progressively more costly, with associated technical difficulties and lower throughput, but provides more information on the biological effects of the compounds needed to guide hit prioritisation and progression. As with any other drug discovery pipeline,

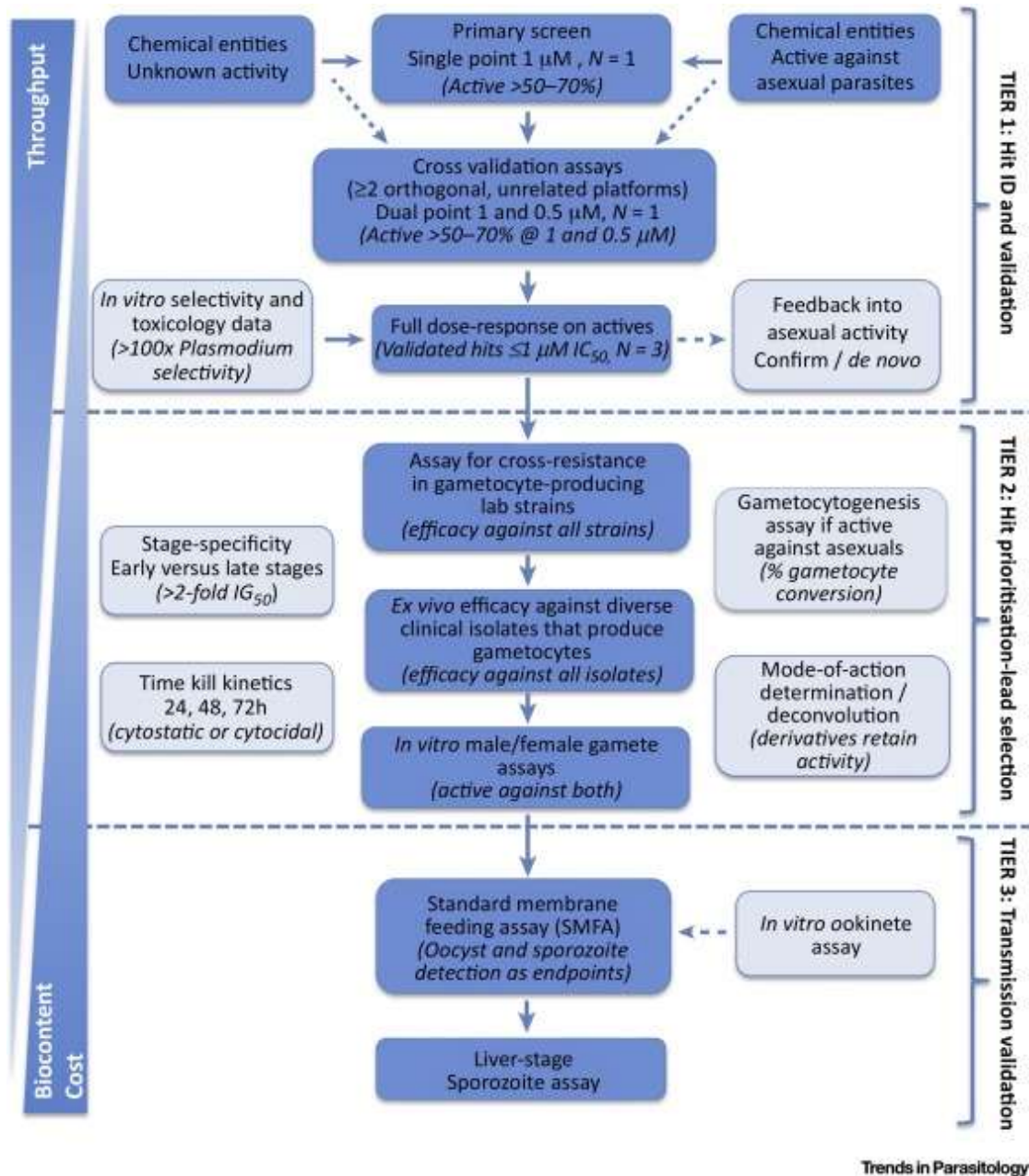


Figure 2.

Proposed Test Cascade for Screening of Transmission-Blocking Antimalarial Compounds. The proposed three-tiered screening cascade provides biologically richer information because compounds progress through each tier with more involved assays, albeit with decreased throughput. Application of strict selection criteria (indicated in italics in each block) guides the progression of compounds through the cascade. The primary cascade is indicated in the centre in dark blue, with parallel (and in some cases optional) investigations indicated in lighter blue blocks.

chemical considerations of the compounds tested are important including lack of pan-assay interference, re-synthesis complexity, reactivity, mutagenicity, safety, etc. Cheminformatic filtering of large libraries (e.g. between tier 1 and

2) is therefore be an important step to eliminate screening irrelevant compounds.

Tier 1: Hit identification and validation

A robust, validated protocol for producing high quality viable *P. falciparum* gametocytes, able to infect mosquitoes, is essential to enable direct comparison of data from different laboratories in a timely manner thereby avoiding duplication and minimising costs [19]. Assays to measure the killing of metabolically hypo-active gametocytes are challenging but different groups have developed several platforms (e.g. [14, 19, 20, 23, 27, 31, 34, 36-40]). In Tier 1 the screening is focussed on activity against stage V gametocytes, ideally with minimal contamination from residual immature stage IV, although an early indication of compounds active against multiple gametocyte stages including immature stages (I-IV) (see tier 2 below), is useful for dual-(or pan)reactivity.

A primary screen is performed using an assay platform (e.g. colorimetric [19, 37], enzymatic, based on stage-specific reporters [36, 38] or high-content imaging [20, 23]), which is amenable to (medium-)/**high-throughput screening** (MTS/HTS). To date, no difference has been seen in activity of compounds tested against wild-type or transgenic parasite lines [36] although different genetic backgrounds should always be taken into consideration in the interpretation of results. To compensate for the limited availability of biological material, primary screens typically employ 384- or 1536-well plate formats (100-1000 compounds / day throughput) and should preferably require ~10,000 gametocytes / well. Non-validated hits are compounds that show $\geq 50\%$ inhibition of mature gametocytes at concentrations of 2-5 μM in the single-point evaluation; 1 μM decreases false hit identification by limiting hit selection of compounds displaying relatively high gametocytocidal activity. To maximise the chance of identifying dual-active hits against, the libraries or single compounds that are screened may have known activity against asexual parasites, alternatively libraries may be screened *de novo* without this knowledge (Box 1). It is imperative to use secondary orthogonal assays as validation counter-screens to provide high-biological content data by using

assays that interrogate different metabolic endpoints. Compounds that are active on several different platforms increase the confidence of hit prediction. These validations therefore importantly filter out false positives from the primary screens and, due to their different readouts, decrease the potential for compound-mediated interference. These are performed on hits using a dual-point screen (hit confirmation >70% inhibition at 1 μ M and >50% at 0.5 μ M) followed by full dose-response evaluation. Validated hits are compounds with an IC_{50} e.g. <1 μ M. Activity against asexual parasites may then be confirmed if prior data are available or else they are tested *de novo*. To prioritise validated hits for progression to Tier 2 of the pipeline, the cost and/or ease of production of each chemical entity is taken into account. Likewise, cheminformatics approaches to computationally prioritise potential hits based on predefined characteristics should be considered. A favourable toxicity profile and high *in vitro* selectivity against parasites are other important filters at this stage. The integrity and purity of the starting **chemical matter** should be verified by conventional analytical means, particularly before downstream assays are performed.

Tier 2: Hit prioritisation

Assays in this tier provide high biological content and additional information to assist in prioritising hits for the next tier. Due to the omnipresent threat of resistance, a critical aspect of Tier 2 is to evaluate the activity of compounds against gametocytes from resistant laboratory strains. The transmission of resistance from asexual blood stages to sexual stages is currently of major interest and gametocytes from known *P. falciparum* resistant strains with diverse genetic backgrounds and well-characterised resistance phenotypes (to known asexual anti-malarials) should be evaluated. More importantly, the activity against sexual stages should also be monitored in an *ex vivo* setting against e.g. circulating African *P. falciparum* parasites from infected patients to provide a confirmation of gametocytocidal action on field isolates.

An *in vitro* male/female gamete assay (e.g. dual gamete formation assay) may be used to initially assess the ability of the compound to have sex-specific activity on gametocytes by measuring male/female gamete formation [30]. An

assay of immature gametocytes will indicate whether the compound exhibits stage specificity, which is an important aspect since the assumption that all compounds targeting asexual parasites will target immature gametocytes does not always hold true [20, 26]. The ability to target all gametocyte stages (I-V) increases the transmission-blocking probability of the compound (Box 1). Kill kinetics provide insight into the speed of action and are useful to ultimately select compounds for combination therapy. Certain compounds with activity against asexual parasites may induce gametocytogenesis as an unwanted side-effect and this could be assessed by gametocyte conversion assays. Investigations into the possible MOA of a compound may be conducted at this level and could include proteome-based target identification strategies (e.g. [24]), cheminformatic clustering [20] or developing MOA deconvolution strategies [40], similar to what has been done for other infectious diseases [41, 42] to retain biological activity within derivatized sets compared to the parent compounds. Assays in this tier characterise the gametocytocidal effects of compounds further and provide an additional level of stringency in prioritising hits for tier 3. The inclusion of field isolates as an early filter is particularly important since it could minimize the downstream attrition rate. However, a lack of information from this tier (e.g. if the biological target has not been identified, or if the kill kinetics are not known) will not preclude progression of a promising compound to the final tier.

Tier 3: Transmission-blocking validation

Correlation of gametocytocidal activity with the ability of compounds to block or reduce human-to-mosquito transmission is still difficult to establish due to a number of issues (Box 2). In Tier 3, the most costly and time consuming assays are performed but they provide the highest biological content. Gamete fertilization and ookinete production in *P. falciparum* is very inefficient *in vitro*, but the development of an assay to fill this gap would be a valuable addition to this tier. Central in tier 3 is the SMFA, which mimics human blood feeding (Box 2). Here, gametocytes are exposed to the test compound and subsequently used to infect *Anopheles* mosquitoes. Dissecting midguts of the infected mosquitoes and quantifying the number of oocysts 7-10 days after infection represents the first end-point. A recent alternative is to use luciferase-

expressing parasites to more rapidly quantify infectivity as bioluminescence from intact mosquitoes [43]. An additional endpoint is to evaluate the salivary glands after 14 days to determine the effect of the compound on the development of sporozoites. These assays are technically challenging but they provide a critical filter before proceeding to an *in vivo* setting. The final step in this tier is to close the loop of the parasite life cycle by evaluating the ability of sporozoites that developed from treated gametocytes to infect human liver cells.

Box 2. Gametocytocidal activity as indicator of reduced mosquito infectivity?

Screens for compounds targeting mature gametocytes remain the most practical way in which indications of transmission-blocking ability can be achieved in a timely and economic way. However, the direct correlation with human-to-mosquito reduction in infectivity (transmission-blocking) is difficult due to a number of constraints:

- 1) There is no direct linear association between numbers of parasites in the different life cycle stages and the transmissibility to mosquitoes [10, 48]. This makes predictions of infectiousness from parasite load difficult. Transmission however, seems highly efficient even at low mature gametocyte densities [49].
- 2) Technically, there is a lack of indicators of gametocyte fitness that can be directly correlated to parasite infectivity and ability to form oocysts in the mosquitoes. An assay specific for male and female gamete formation provide an indication of compound effects on gametocyte infectivity [30], however, this adds complexity to the design of a transmission-blocking pipeline. This also raises the issue of whether there is a need for compounds to target both forms of gametes or potentially only female or male gametes.
- 3) Gametocytocidal activity as the endpoint can result in false negatives or false positives if a single assay platform is used. The use of orthogonal assays in a gametocytocidal screening cascade should increase assay predictability and consider reversible (static) actions. Gamete assays in association with SMFA data (see point 4) may additionally indicate compounds that prevent mosquito infection by targeting gametes (e.g. pyrimethamine)[43], rather than compounds purely affecting gametocyte viability. However, the very narrow timeframe available to target gametes is challenging.
- 4) SMFA is still considered the definitive checkpoint for transmission-blocking capability but this assay is associated with marked technical variability and challenging experimental settings. In terms of assay readouts, IC_{50} values have been reported to vary in rank order as well as in amplitude (180-fold variation) in different published platforms for gametocyte activity and SMFA activity. On the other hand, several

technical advances have led to increased capacity and performance in SMFAs [43, 50].

- 5) Predicting successful transmission-blocking activity by monitoring clinical duration of gametocytaemia, gametocyte clearance rates and gametocyte infectiousness should be considered [51].

Translatability of the *in vitro* studies revealing transmission-blocking potential

Once potencies (IC₅₀s) of leads and drug candidates are compiled in the various transmission assays of the test cascade, it is key to understand how these data will translate into an effective dose administered to humans to ideally block or at least significantly reduce transmission of the disease to other humans. In the case of a dual-activity combination (Box 1), it is anticipated that the driver will be the free concentration available in human blood and effective to treat patients. Ideally, the concentration to block transmission should not be higher than the one needed to cure a patient to prevent increasing the dose of the treatment to meet the goal of transmission-blocking (Box 1). Similarly, safety margins will be derived using the treatment dose rather than the one necessary to block transmission. In the development of a transmission-blocking only drug, safety will become the main driver after transmission-blocking potency. In both approaches, data from *in vitro* assays could be compared with those obtained from relevant animal models such as the mouse-to-mouse *Plasmodium berghei* transmission model (despite the difficulty of using results to model the human parasite transmission dynamics) [44-46], from *ex vivo* studies using blood from controlled infected volunteers (challenge model) and from patients receiving drugs and using this blood in SMFAs.

Conducting large and very costly clinical trials for transmission-blocking, similar to the Burkina Faso Coartem (Artemether - Lumefantrine) trial [47] is extremely challenging. Instead, the acquisition of clinically-relevant transmission data post-launch i.e. along with phase IV pharmacovigilance studies is currently favoured. However, this supports the view that transmission-blocking is only an additional asset of a curative treatment and

does not enable the early development of dual-active and transmission-blocking only compounds. For the latter two, translatability is key to guide the medicinal chemistry process and would require development of e.g. unique pre-clinical evaluation models. It is therefore essential to consider what **pharmacokinetic/pharmacodynamic** (PK/PD)-based modelling approaches will be useful in different transmission settings to set criteria earlier on in development of transmission-blocking drugs.

Concluding remarks

The global focus on malaria elimination has opened an opportunity for the identification of compounds with the ability to block human-to-mosquito transmission. The recent development of several robust HTS platforms to identify compounds with gametocytocidal activity provides a rich starting point to this goal but requires the design of a rational screening cascade. The test cascade outlined here provides a logical flow of biologically rich screening abilities whilst maintaining strict orthogonal validation and progression analysis. The endpoint of the cascade is the experimental confirmation that the gametocytocidal activity translates into the ability of the compound to block parasite infection of mosquitoes. The design of a rational cascade still leaves open the main questions (see 'Outstanding questions') on the value and use of the compounds that have been identified, the choice of prioritizing compounds with preferential or sole gametocytocidal activity, and, last but not least, the form in which such compounds will be deployed in the fight against malaria. Ultimately, the contribution that transmission-blocking anti-malarial drugs can provide towards malaria elimination as an overarching strategy should now be interrogated and could surpass all other more elusive strategies including mosquito control and vaccines.

Which parasite stages are suitable transmission-blocking targets? The long maturation of *P. falciparum* gametocytes makes them suitable drug targets, and circulating mature gametocytes are pharmacologically available. Targeting nondividing parasites will also decrease the risk of developing resistance. Potential benefits of targeting gametes in the

mosquito blood meal are offset by the demanding requirements for drugs whose action is limited in time and space.

Should biological targets differ between asexual and sexual stages? Compounds that have a different MOA in asexual and sexual parasites will be advantageous in minimising resistance, but should not be viewed as an essential attribute of a transmission-blocking drug.

What should a transmission-blocking compound look like? An optimal TCP should favour compounds with a long pharmacological action, but will depend on the rate of asexual parasite clearance in the combination; gametocyte-stage specificity; and efficacy in the vector. It should also address which *in vitro* activities translate to good activity *in vivo*.

How will clinical trials for transmission-blocking compounds be performed? This is possibly the biggest challenge to develop transmission-blocking-only compounds. It requires unique testing strategies [*ex vivo* studies from human volunteers (challenge model) and extensive SMFAs] and has to address submicroscopic gametocyte loads in endemic populations.

How will such compounds be used? Ethical issues arise in this context, since the potential community benefit of malaria elimination by blocking transmission may outweigh potential risks. The ideal strategy is to develop a combination of curative and transmission-blocking agents. Whether this will prove as effective as the individual compounds, given notable PD complexities, including dosage challenges and stability, remains to be clarified. Since asymptomatic carriers significantly contribute to parasite transmission, the question arises as to whether these compounds can be used alone in MDA campaigns to meet the eradication agenda.

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Resources

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Glossary

Chemical matter: chemical compounds (synthetic or derived from natural products) with potential inhibitory action either on whole cell or target level; originating from either small panels of a few compounds and their derivatives or from large libraries of usually diverse chemical entities.

Elimination: the point at which the incidence of infection with *Plasmodium* parasites is no longer detected within a defined geographical area and no local malaria cases are reported.

Eradication: a permanent intervention whereby infections cease and re-emergence or re-establishment of transmission no longer occurs.

Gametocytes: intra-erythrocytic sexual-stage parasite forms that are transmitted from humans to the *Anopheles* vector, thereby mediating human host to mosquito vector transmission. These develop through 5 distinct stages; immature gametocytes (stages I-IV) sequester in bone marrow whilst stage V gametocytes circulate; only mature stage V gametocytes are infectious, although no marker of maturity to predict infectiousness to mosquitoes is currently available.

Gametes: exo-erythrocytic sexual-stage parasites that are induced following activation in the mosquito midgut. One male and one female gamete subsequently fuse to form the fertilised zygote.

High-throughput screening (HTS): rapidly assaying large numbers of potentially active compounds in parallel, typically against single phenotypic or target-based end-points.

Mode-of-action (MOA): a particular and distinct biochemical pathway or specific (usually protein) target identified as the mechanism by which chemical compounds effect their inhibitory action.

Mass drug administration (MDA): strategies through which target populations are treated with a single drug to achieve malaria transmission reduction. Since this takes place on population level, administration occurs irrespective of disease symptoms present in an individual.

Oocysts: the parasite developmental stage within the basal lamina of the mosquito midgut epithelium prior to the rupture and release of sporozoites, which subsequently migrate to the salivary glands.

Ookinetes: the infective motile parasite stage where meiosis occurs and responsible for traversing the mosquito midgut prior to invasion and development between the epithelium and basal lamina.

Orthogonal assay: an assay that is performed following a primary screen, which utilises a different reporter or end-point readout to distinguish true hits from false-positive observations.

Population bottlenecks: During the parasite's life cycle, population size decreases dramatically at two points, enabling transmission between human hosts and mosquitoes: 10-100 parasites (bottleneck 1) initiate infection in humans where parasite numbers can increase to billions, but only ~1000 (bottleneck 2) are required to enable transmission to mosquitoes.

Pharmacokinetics/pharmacodynamics (PK/PD): PK describes temporal investigation of drug concentrations after *in vivo* dosing; PD describes the resultant *in vivo* effect of a drug dosis.

Polypharmacology: The ability of a single chemical entity to elicit more than one biological response, for instance, targeting both asexual and sexual forms of malaria parasites. These could also have different modes-of-action in each scenario.

Sporozoites: motile forms of *Plasmodium spp.* that infect the human liver following transmission during feeding of female *Anopheles* mosquitoes.

Standard membrane feeding assay (SMFA): an assay whereby the *Anopheles* mosquito vector is infected with gametocytes and the development of oocysts is monitored to evaluate the effect of a transmission-blocking candidate.

Target product profile (TPP): the desired minimally acceptable characteristics required for combination medicines.

Target candidate profile (TCP): the proposed optimal and minimally acceptable characteristics for clinical candidate molecules.

Transmission-blocking: the prevention of parasite transmission between human host and the mosquito vector (or *vice versa*) by drug-based intervention or other means.

Abbreviations:

HTS: high-throughput screening; MDA: mass drug administration; MMV: Medicines for Malaria Venture; MOA: mode-of-action; MTS: medium-throughput screening; PD: pharmacodynamic; PK: pharmacokinetic; SMFA: standard membrane-feeding assay; TCP: target candidate profile; TPP: target product profile.

Table 1. Summary of chemical libraries screened for mature gametocytocidal activities.

Gamete stage (day)	Assay	Assay parameters	Assay format (number of wells)	Library ID ^a	Number of compounds	Hit criteria % inhibition	Hit rate	Examples: Hit ID & activity (IC ₅₀)	Hit class/targets	Refs
V (d13)	AlamarBlue	72h 20% serum	1536	LOPAC	1280	>75% @ 1.8µM	7/1280 2 selective	Antabuse (0.25 µM) CyPPA (1.17 µM)	Aldehyde dehydrogenase KCa2.2	[37]
73% IV/IV (d12)	AlamarBlue	72h 20% serum	1536	1) NIH NPC 2) MIPE	4265 550		27/5215 21 selective	NSC174938, Torin2, NVP-AuY922, maduramicin, narasin (3-50 nM)	Kinases (e.g. PI3K) Dibenzazepines Ionophores D2 agonists TDPE Hsp90 RPPK ATCase	[24]
IV/IV (d11)	AlamarBlue	72h 20% serum	96	St Jude	650	>50% @ 10 µM	9% (23/260)	SJ000030570 (61 nM)	Diaminonaphthoquinones Dihydropyridines Bisphenylbenzimidazoles Cabazoleaminopropanols Iminobenzimidazoles	[32]
V (d15)	ATP	48 h 10% Albumax II	384	TCAMS	13 533	1: >90% @ 5 µM 2: >50% @ 1 µM	373/13 533 hits 98 selective	TCMDC-137476 TCMDC-123885 TCMDC-123792	Quinacrine-like KCa2.2 PfATP4	[33]
V (d15)	SYBR Green / exflagellation	48 h 10% serum	96	JHU CCL	1 500	>70% @ 20 µM	25/1500	Anastrozole (600 nM)	Antiseptics Antineoplastics antiprotozoals	[29]
91% V (d12)	MTR Red, high content imaging	72 h 4.3% serum	384 / 1536	1) GNF malaria box 2) Broad DOS	3855 9886	>70% @ 1.25 µM >30% @ 2.5 µM	4% (145/3855) 0.25% (25/9886)	22 cpds <100 nM 13 cpds <5 µM	Carbamazidethiureas Naphthoquinones Diozonaphthalen-acetamides Tetrahydroisoquinoline-4-caboxamides 2-furancarboxamides	[20]
IV/IV (d 8)	Luciferase reporter (NF54 ^{Pfs16})	72 h 5% serum	384	1) ERS_01 2) GDB_04	4760 4900	>50% @ 4 µM >50% @ 10 µM	12/4760 (0.25%) 30/4900 (0.61%)	9 confirmed 30 confirmed (e.g. SN00771077 (1204 nM))	ND	[28]

^a Lopac: Library of pharmacologically active compounds (SigmaAldrich); NIH NPC: Chemical Genomics Centre Pharmaceutical Collection; MIPE: NIH internal collection of kinase inhibitors; TCAMS: Tres Cantos Anti-malarial Set ; JHU CCL: Johns Hopkins University Clinical Compound Library; Broad DOS: Diversity oriented synthesis library; ERS_01: commercially available diversity set, Eskitis; GDB_04: diversity set on 1225 scaffolds from Compounds Australia.

MTR= mitotracker; TDPE=Tyrosyl-DNA phosphodiesterase; RPPK=phosphoribosyl pyrophosphate synthetase; ATCase = aspartate carbamoyltransferase; KCa2.2 = Ca²⁺ dependent K⁺ channel; PfATP4 = P-type cation-ATPase, Gam: gametocyte, ND: not determined / indicated