CellPress
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Trends in
Parasitology

Review

Transmission-blocking drugs for malaria elimination

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Preventing human-to-mosquito transmission of malaria parasites provides possible solutions to interrupt the malaria parasite life cycle for malaria elimination. The development of validated routine assays enabled the discovery of such transmission-blocking compounds. Currently, one development priority remains on combinations of dual-active compounds with equipotent activity against both the disease-causing asexual and transmissible, sexual erythrocytic stages. Additionally, transmission-blocking compounds that target gametocyte-specific biology could be used in combination with compounds against asexual parasites. In either case, preventing transmission will reduce the risk of reinfection and, if different processes are targeted, also curb the spread of drug resistance. Here, we provide an updated roadmap to the discovery and development of new antimalarials with transmission-blocking activity to guide drug discovery for malaria elimination.

Highlights

Malaria burden can be demonstrably reduced by blocking parasite transmission between the human host and mosquito vector.

Antimalarial drugs that target blood-stage parasites as well as block onward parasite transmission will be useful for elimination strategies.

Transmission-blocking activity that is associated with targeting different biological processes, or showing polypharmacology, will have obvious advantages in combination strategies to protect blood-stage actives from resistance transmission.

Candidate drugs with equipotent activity against blood stages and transmissible stages have been identified and show promise in development as transmission-blocking strategies.

Malaria parasite transmission

Antimalarial drug discovery is driven by the need for new drugs to treat infections and save lives in a constantly evolving drug-resistance context. However, the ability of such new drugs to also block transmission and stop the spread of the disease is of utmost importance to malaria **elimination** (see Glossary) strategies in countries working towards zero infections. Besides the essential ability to cure patients of disease [target candidate profile 1 (**TCP-1**)], new antimalarial candidates can fit other **TCPs**, including preventing human-to-mosquito transmission (**TCP-5**), and/or prophylaxis (**TCP-4**). These TCPs require **compounds** with long-lasting activity as a priority [1,2].

Targeting transmission is based on the conceptual logic associated with targeting parasite stages at their population bottlenecks (Figure 1). Indeed, <100 of the 10^3 **sporozoites** of the human malaria parasite, *Plasmodium falciparum*, available in one mosquito bite can seed an infection in humans to initiate exoerythrocytic asexual schizogony [3]. Human-to-mosquito transmission similarly requires few parasites, with only 10^3 of the 10^8–10^9 mature, sexual stages (falciform **gametocytes**) circulating in the blood of highly infected patients required to be transmitted to the next feeding female *Anopheles* mosquito taking a ~2 µl blood meal (Figure 1, [4]). This bottleneck is further evident as, out of the small number of 100 ookinetes eventually maturing in the mosquito gut, only 3–5 **oocysts** are formed in the infected mosquito to provide the infective sporozoites. Altogether, these parasite numbers are massively reduced compared with the ~10^{11} **asexual blood stage** (**ABS**) parasites that can be reached in an uncontrolled infection, resulting in severe malaria [5,6].

The advantages of blocking malaria transmission to enable malaria elimination

Antimalarial drugs that target human-to-mosquito transmission would have the obvious advantage of reducing the parasite prevalence in an endemic population by clearing gametocyte

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reservoirs and/or preventing onwards transmission. Such **transmission-blocking** activity would require eliminating or inactivating individual mature gametocytes. This activity should ideally persist after the malaria symptoms, caused by the ABS parasites, have been alleviated. This will prevent the mosquito population from being infective and suppress any subsequent occurrence of malaria infection.

Transmission blocking would not only be important to prevent onwards spreading of gametocytes by symptomatic patients but should also target gametocytes in asymptomatic carriers. Up to 84% of persistent transmission is driven by asymptomatic, microscopically detected carriers of mature gametocytes [7], irrespective of age group and even in low-transmission settings [8]. Therefore, the introduction of transmission-blocking antimalarials will be compromised unless the ~30% of infectious individuals that can carry submicroscopic gametocytes are identified and treated or mass treatment in undetected scenarios is applied [9,10]. This reasoning is at the basis of the use of low-dose primaquine during localized mass drug administration campaigns in transmission hotspots. Such a strategy is under evaluation in some African countries moving towards malaria elimination [11]. Moreover, chronic infection can also result in a high gametocyte carriage, representing a significant source of onwards transmission [12], and during the dry season in low-transmission conditions, gametocytes are maintained in the population due to an increased rate of gametocyte conversion [13].

Possibly the most important contribution of compounds with transmission-blocking activity would be to protect molecules with ABS activity by delaying the spread of resistance. Compounds will generally do so by reducing the parasite reservoir, whereas protection of the ABS activity against resistance will also be specific should compounds be active on different targets in the two stages. Drug-resistant *P. falciparum* parasites are inevitably selected during the ABS proliferation phase [14], but once resistance has developed it is easily transmitted, as evidenced by multiple examples from the past. In fact, when experimentally tested, gametocytes exhibited the same resistant trait that had arisen in the asexual stages [15–17]. An exception to this rule, where mutations render ABS parasites resistant to drugs while this resistance phenotype is not transmitted, was seen with atovaquone. Here, ABS resistance resulted in blocking transmission [18], and the mutation was reported to be lethal for the sexual stages in the mosquito. However, this exception may be unique to atovaquone, killing ookinetes due to its specific **mechanism of action** (**MOA**), targeting respiration in these stages.

P. falciparum has a gametocyte maturation process of 8–12 days in humans, uniquely long among the human malaria parasite species, and gametocytes become progressively insensitive to antimalarial compounds/drugs during their maturation [19]. Importantly, because not all ABS schizonticides can efficiently clear immature (stage I–IV) gametocytes (e.g., antifolates, atovaquone [19]), killing these early sexual forms with transmission-blocking compounds is essential as this would reduce, if not abrogate, the eventual formation of mature gametocytes (stage V). A drug combination that is unable to kill immature gametocytes whilst targeting mature gametocytes will therefore have potentially a particular problem with resistance transmission as was seen with the sulfadoxine–pyrimethamine combination [20].

Compounds with transmission-blocking activity can be combined with one (or two) ABS actives (with required activity on immature gametocytes), as exemplified by clinical trials where primaquine was added to current artemisinin combinations [21]. Although primaquine displays poor activity against asexual and sexual parasites *in vitro*, this drug is efficacious *in vivo* once metabolized by the host CYP450-2D6 enzyme [22] and thus can be considered as a dual-active compound with transmission-blocking activity. The main drawback of a dual-active compound is that, in the

Glossary

Asexual blood stage (ABS): asexual, intraerythrocytic parasites undergoing growth and proliferation (schizogony) and morphologically described to progress from inactive ring-stage parasites to growing, metabolically active trophozoites followed by the actively dividing schizonts, leading to the production of ~28–32 daughter merozoites which are released to reinfect new erythrocytes.
Compounds: chemical compounds (synthetic or derived from natural products) with potential inhibitory action either on the whole cell or at the target level; they originate from either small panels of a few compounds and their derivatives or from large libraries of usually diverse chemical entities.
Dual activity: compounds with the ability to target both asexual and sexual stages of the parasite are described as being dual active. Such compounds would be able to alleviate a patient's symptoms whilst simultaneously preventing onward parasite transmission.
Dual gamete formation assay (DGFA): the simultaneous measurement of a compound able to block formation of male and female gametes, using two directed assays against gametocytes, the precursors of each of these forms.
Elimination: the point at which the incidence of malaria infection is no longer detected within a defined geographical area and no local cases are reported.
Gametes: male and female exoerythrocytic sexual-stage parasites that are induced following activation of the gametocytes in the mosquito midgut. One male and one female gamete subsequently fuse to form the fertilized zygote.
Gametocytes: intraerythrocytic, sexual-stage parasites that are transmitted from humans to the *Anopheles* vector in which they transform into gametes, thereby mediating human host to mosquito vector transmission. These develop through five distinct stages. Immature gametocytes (stages I–IV) sequester in bone marrow, whilst stage V gametocytes circulate; only mature stage V gametocytes are infectious.
High-throughput screening (HTS): rapidly assaying large numbers of potentially active compounds in parallel, typically against single phenotypic or target-based endpoints.

vast majority of the cases, it will block the same target in both the asexual and sexual-stage parasites, hence favoring the transmission of the resistance that might appear against the former stage.

Due to this, several parasitologists recommend the search for transmission-blocking 'specific' compounds that target gametocyte-specific biology. Molecules of this type, being inactive against the asexual stages, will necessarily then have to be combined with two different ABS actives protecting each other from insurgence of resistance. In such a triple combination, the activity of the transmission-blocking compound on the immature and mature gametocytes will prevent parasite transmission and, in the likely case that this compound has limited or no activity against the immature sexual stages, the development of immature gametocytes will have to be prevented by at least one of the ABS compounds. As the target of the transmission-blocking compound will not be subject to resistance selection, adopting such a triple combination will have the advantage of a predictably extended life of the therapy in the field compared with a dual combination using a dual-active compound.

Although the development of a transmission-blocking active, without ABS activity, would be faced with multiple developmental challenges (Box 1), these should be balanced in view of the biological advantages that relying on such a compound would provide. Even though the development of dual-active compounds can be considered easier, their limitation regarding potential resistance containment has to be considered. Moreover, the cost-effectiveness of identifying and developing dual-active compounds (hitting the same biological target in different parasite stages) should not preclude the possibility of identifying transmission-blocking specific compounds that will target the transmission population bottleneck.

The long-term advantages of blocking human-to-mosquito transmission are therefore clear, and progress towards the development of transmission-blocking activity in antimalarials has been remarkable [23]. Here, we provide an updated framework outlining the requirements for discovery and development of antimalarials with transmission-blocking activity, highlighting the unique and potentially targetable biology associated with malaria transmission.

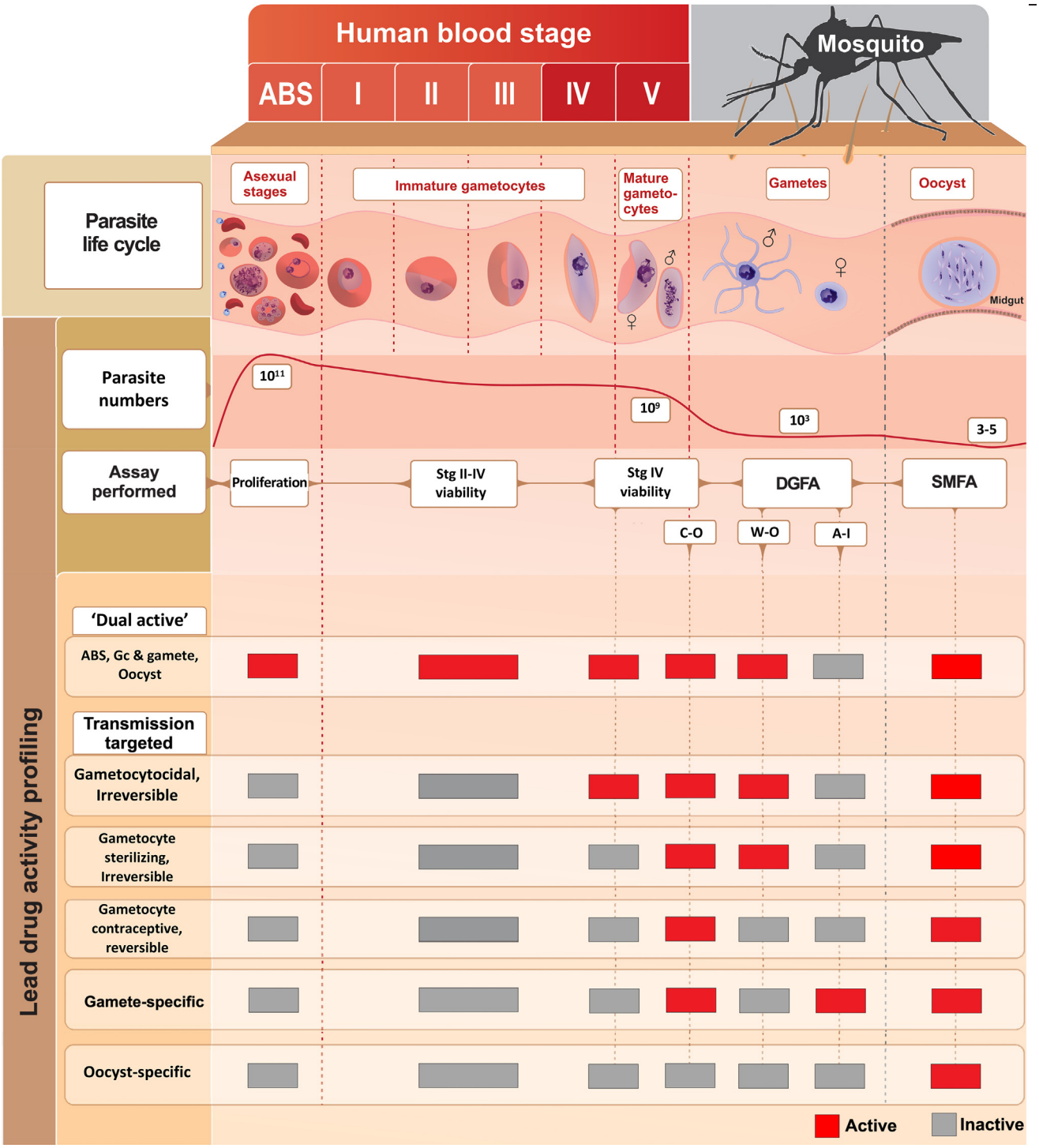
Defining transmission-blocking activity

The discovery objective for transmission-blocking entities is defined as identifying compounds active against the transmissible stages of *Plasmodium in vitro*. Empirical proof of such activity is now routinely obtained for any candidate antimalarial where ABS activity has already been defined, marking such compounds with '**dual activity**' when they also target transmissible stages (Figure 1, [19,24–28]). Demonstrating transmission-blocking activity of a compound, however, typically includes multiple endpoints to show a reduction in several of the developmental stages associated with transmission. These endpoints include direct counts of gametocytes and functional evaluation of **gametes** and oocysts (Figure 1).

Technological advances have resulted in assays now available to test any of these developmental stages for *P. falciparum*, some of which are amenable for medium-/**high-throughput screening** (MTS/**HTS**). In contrast to ABS activity, that usually measures the inhibition of cell proliferation, gametocytocidal activity relies on targeting differentiated parasites and therefore is measured with viability indicators including metabolic readouts [19,25,26,29–40] or reporter lines [35,40–44]. In these assays, primary indicators of transmission-blocking activity should be gametocytocidal activity on both immature (stage I–IV) and mature (stage V) gametocytes [28,36,45,46].

Gamete assays tend to show sex specificity [38,47], with female gametes only sensitive to about a quarter of compounds active on the flagellated, replicating male gametes. Additionally, comparing

Mechanismofaction (MOA): a particular and distinct biochemical pathway or specific (usually protein) target identified as the mechanism by which chemical compounds effect their inhibitory action.
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.
Orthogonal screen: an assay that is performed following a primary screen, which utilizes a different reporter or end-point readout to distinguish true hits from false-positive observations.
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 dose.
Polypharmacology: the ability of one compound to target more than one protein target or different biological processes.
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 via feeding through an artificial membrane covering a vessel containing infected blood, and the development of oocysts is monitored to evaluate the effect of a transmission-blocking candidate.
TCP: target candidate profile, as defined by the Medicines for Malaria Venture (www.mmv.org).
TCP-1: compounds targeting asexual parasite proliferation and thereby being curative of disease symptoms.
TCP-4: compounds targeting hepatic schizogony and thereby having prophylactic function.
TCP-5: compounds preferably targeting all stages of gametocytes and preventing gamete and oocyst/ookinete formation and thereby having transmission-blocking activity.
Transmission blocking: the prevention of parasite transmission between the human host and the mosquito vector by drug-based intervention or other means.



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gametocytocidal versus gametocidal activity can indicate if a compound kills or only compromises a gametocyte to the point where it only affects processes important for fertility that are revealed only at later stages (Figure 1, [23,48]). Validating transmission-blocking activity depends on evaluation of reduced ookinete/oocyst formation in mosquito experimental infections. *P. falciparum* ookinetes are so far impossible to routinely assay in screening campaigns *in vitro*, but these mosquito stages are more accessible in the *Plasmodium berghei* model, which can thus provide an indication of targetable biology in these stages. Pragmatically, proof of loss of functional sporozoites would be required for the validation of this activity since, in rare circumstances, compounds may lead to the formation of oocysts containing nonfunctional sporozoites and thus present as false negatives should oocyst counts be the endpoint.

Ultimately, a transmission-blocking activity that only works in mosquito-stage parasites (e.g., only gametocidal or sporontocidal) is less favored than one able to also target gametocytes in human blood (by killing them or making them permanently infertile), where these stages are better accessible to pharmaceutical intervention (Box 1). A drug that is unable to kill *P. falciparum* gametocytes in human blood will need an extraordinarily long human plasma half-life and potency to be effective against the later parasite stages in the mosquito [49]. The only alternative would be direct contact exposure of mosquito populations to such compounds to target oocyst and sporozoite formation [50] or compounds that alter, during gametocyte differentiation, a developmental program that is essential for parasite viability in the mosquito.

Targeting the Achilles heel of the transmission stages

The divergent biological processes associated with transmissible stages of the parasite provide several opportunities for the discovery of interventions. Consequently, ample repertoires exist of gametocyte- and male/female-specific proteins that are active predominantly during these stages (Box 2). The relevant biology associated with transmission extends from differentiation of immature gametocytes from their asexual progenitors, most obviously from stages II–III of development [51] but accentuated in mature gametocytes [51,52], male and female gametes and ookinete/oocysts [53–55].

Dual-active chemotypes mostly target the same process in multiple stages and include inhibitors of kinases (e.g., [26]), protein biosynthesis [56,57], the proteasome [58], protein modification and membrane trafficking, phospholipid metabolism [59], and ion homeostasis [19,46,60], which all kill both ABS and transmissible stages (Table 1). The enrichment towards protein metabolism, whilst not surprising, is interesting in the face of reported translational repression of parasite transcripts required for female gamete to zygote transition [52], contributing to our understanding of the essential processes required for fertilization. Additional processes with importance in transmissible stages include signaling pathways [61–63], mitochondrial respiration [45,64], and the epigenetic landscape ([45,65–68],Box 2). Dual-active compounds include front-runner antimalarials that show equipotent activity against ABS and immature and mature gametocytes: Cipargamin (KAE609 – targeting PfATP4), M5717 (DDD498 – targeting translation/elongation

Figure 1. Key profiles of transmission-blocking activities of antimalarial candidates. To target human-to-mosquito transmission of the human malaria parasite *Plasmodium falciparum*, antimalarial candidates are profiled on multiple life cycle stages. These include vastly different parasite numbers, from 10^{11} asexual blood stage (ABS) parasites, to 10^{9} mature, stage V gametocytes, of which only 10^{3} are transmitted to the mosquito. In those, typically, only three to five oocysts form in natural infections, whilst laboratory models can produce 15–30 oocysts. *In vitro* assays are available for each life cycle stage, measuring proliferation of ABS and viability of immature (stages II–IV) or mature (stage V) gametocytes. The dual gamete formation assay (DGFA) simultaneously determines formation of male and female gametes, in different formats [carry-over when stage V gametocytes are treated (C-O), and drug then washed-out (W-O)] or in add-in format directly during gamete induction (A-I). The standard membrane feeding assay (SMFA) provides the validation of transmissionblocking and loss of fertility in the parasites by determining reduction/blocking of oocyst formation. Compounds can be considered active on several of the stages (red blocks = active compound; gray = inactive), and the combination of the activity profile allows classification of compounds as having activity against multiple stages or having transmission-targeted activity.

Box 1. Developmental challenges for transmission-blocking activities

Transmission-blocking-driven primary screens

The key objective of transmission-blocking-driven primary screens remains the identification of gametocytocidal compounds based on the divergent biology associated with these stages. The choice of starting point is mostly technology platform driven but, unlike ABS-driven campaigns, HTS transmission-blocking screens cannot rely on a single assay to identify all transmission-blocking molecules. An HTS that incorporates the complexity of the cell biology associated with all the transmission stages is currently lacking. A primary screen on stage V gametocytes will therefore require transmission-blocking validation of hits on subsequent stages, and comparatively, a screen driven by inhibition of gamete formation will have to retrospectively prove efficacy on stage V gametocytes to allow further development.

Combinations of compounds

Compounds with potent transmission-blocking activity, and targeting biology unique to the transmissible stages, can be conceivably combined with ABS actives, provided that the ABS actives hit a different target and kill immature gametocytes. That combination would protect the ABS actives and prevent resistance spread. However, development would require combination of two ABS actives (to prolong resistance development) with a transmission-blocking active. Such triple combinations would be associated with increased development costs and pharmacological complexities to match PK/PD profiles for the TCP required, including safety assessments, but clear clinical advantages exist as exemplified in triple combination therapies against mycobacteria.

Compounds with singular transmission-blocking activity?

The use of a 'transmission-blocking only' compound holds promise to greatly decrease the vast parasite reservoir in asymptomatic individuals within whole populations in a mass drug administration scenario or in targeted delivery to gametocyte carriers if they could be successfully identified. Such a strategy will have to be used in parallel with treatment for patients and will not directly benefit the asymptomatic gametocyte carrier, but it will protect the community. Development of compounds with only transmission-blocking activity does not hold the same weight as dual actives since limited resource requirements will be aimed towards compounds with therapeutic benefits, and repurposing compounds with known safety profiles for transmission-blocking could rather be explored. Innovations such as developing a transmission-blocking active as a 'slow-release formulation' [100], will allow targeting of gametocytes along the entire, prolonged maturation period, with the drug still available as gametocytes mature. Ultimately, transmission-blocking actives may indeed have a role in more cost saving and efficient post-treatment approaches, targeting individuals from which the ABS population has been diminished whilst ensuring that any escaped gametocytes are cleared efficiently.

Human dose prediction for transmission-blocking activity

Insectaries associated to NSG (SCID) mouse facilities are now available to support *in vivo* studies of transmission-blocking efficacy. In the case of a dual ABS-transmission blocking (TCP-1+TCP-5) compound, the effective concentration (the ABS *in vivo* EC_{50}), together with the human PK prediction, will determine the dose. If the potency is higher for sexual-stage parasites than for the ABS ones, the dose prediction would not be compromised; however, if the opposite is true (as is mostly the case), the opportunity to increase the dose to match the transmission-blocking requirements will be determined by the curative dose and therapeutic margin of the compound. In the case of a triple combination with a TCP-5-only compound, this will not be the case and this scenario will have more flexibility.

factor 2), and MMV048 (targeting phosphatidylinositol-4-kinase, PI4K, Table 1). The question of equipotency is indeed essential (Box 1), with modeling of transmission-blocking efficacy based on natural infection intensity confirming M5717 as the most attractive development candidate in this regard [69].

From a target perspective, compounds that show **polypharmacology** are enticing and would have the advantage of reducing the risk of resistance development [70]. To this end, it is important to understand whether one compound hits the same target in asexual and sexual stages or different stage-specific targets, something usually only established with some hindsight. The cGMP-dependent protein kinase (PKG) is a clear example of the first scenario, being targeted in blood in asexual and sexual parasites and in mosquito stages, a situation that opened the development of several antimalarial compounds [63].

The genes encoding potential antimalarial drug targets are generally identified from whole-genome sequencing of mutated parasite clones surviving sublethal drug exposure in ABS [71–74].

Box 2. Unique, targetable, biology to block malaria parasite transmission

The developmental branchpoint between indefinite multiplication cycles or the terminal differentiation into the sexual stages is the entry point to the different biological processes of asexual- and sexual-stage parasites. Several processes are conceivably targetable, predominantly or specifically in gametocytes:

(i) Commitment to sexual differentiation requires specific epigenetic molecular players (HP1, GDV-1, AP2-G), and interference with these can massively shift the differentiation of *P. falciparum* towards alternative, virtually pure asexual- or sexual-stage populations [85]. Recent controlled *P. falciparum* experimental infections in volunteers confirmed old observations that commitment to gametocytogenesis can occur very early in infections, even in the first generation of blood-stage merozoites emerging from the liver [86], which, in principle, makes it conceivable to identify molecules able to epigenetically induce the early abortion of the entire blood-stage asexual proliferation.

(ii) *P. falciparum* immature gametocytes actively remodel the infected erythrocyte [87], suggesting that this contributes to exposing antigens and establishing regulatory interaction with host cells [88]. A mechanistic link to the ability of immature gametocytes to reach and establish themselves in the protective bone marrow microenvironment for maturation is far from being demonstrated, but targeting gametocyte sequestration in addition to circulating, mature gametocytes would have a dramatic impact on parasite transmission. In addition, an underestimated aspect of gametocyte sequestration in the bone marrow regards the possible consequences of the long-lasting presence of maturing gametocytes in the extravascular compartment of this organ [89] and, as suggested by *in vitro* evidence, their development inside cells of the erythroid lineage [90] or their interplay with bone marrow mesenchymal cells [88]. In fact, studies investigating whether the *P. falciparum* gametocyte nurseries cause any deleterious effects to bone marrow physiology might question the current dogma that sexual stages are not pathogenic in the human host.

(iii) Inducing the blood circulation of erythrocytes containing immature gametocytes is predictably leading to clearance by the spleen, as these cells are mechanically more rigid than those containing the mature stage V, the only sexual stages normally seen in peripheral blood. Preventing the developmental switch in cell mechanical properties that make these mature gametocytes highly deformable would make them susceptible to being trapped by the spleen, as in the case of the immature stages. Progress has been made to dissect the molecular machinery and to identify inhibitors regulating these cell mechanical properties [91], which may open the way to developing drugs acting on these gametocyte-specific processes.

(iv) New permeation pathways (NPPs) ensure nutrient uptake in asexual parasites and immature gametocytes, whereas their activity declines during maturation under cyclic AMP (cAMP) signaling regulation [92]. Besides the insights into potentially targetable processes, this observation contributes to explain the progressively decreasing susceptibility to drugs which accompanies gametocyte maturation [19]. It also suggests that forced opening of NPPs in drug-treated stage V gametocytes may narrow the commonly observed gap in IC_{50} values between asexual and sexual stages.

(v) Intracellular signaling, including Ca^{2+} and cGMP signaling involving kinases such as Ca^{2+}-dependent protein kinases (CDPK4 [61], CDPK1 [62]) and PKG [63], respectively, is essentially important in the transition to gametogenesis. Since CDPKs are exclusive to *Plasmodium*, these indeed provide unique opportunities for selective inhibition of parasite viability and transmission [93].

(vi) Energy metabolism indeed remains a potentially rich source of targets due to the switch to oxidative metabolism in gametocytes from the fermentative metabolism associated with asexual blood stages [77]. This manifests as upregulation of mitochondrial proteins [94] and associated mitochondrial complexes [95]. Type II NADH:ubiquinone oxidoreductase and flavoprotein subunit of complex II for instance are essential to gametocyte survival, and reliance on oxidative phosphorylation is pronounced in mosquito stages, explaining the activity of atovaquone to block transmission.

In the few cases investigated [15], these mutations transfer resistance to the isogenic gametocytes, an observation that challenges the development of such compounds. More rarely, for example, for ganaplacide (KAF156 – targeting protein secretion) and the resulting mutations in *pfcarl*, the selected trait is considered a marker/mechanism of resistance rather than a target for drug activity. The identification of target candidates in gametocytes that are not transferred from ABS is more complicated and relies on alternative, protein-based strategies [28,75] due to the nonproliferative nature of these parasites.

Roadmap to discovery and development of transmission-blocking actives

With the caveat of the absence of a systematic analysis, most hits from ABS HTSs and most of current antimalarial drugs and lead compounds are inactive on the sexual blood stages, particularly the mature gametocytes [19,35,45]. Assays for ABS hits are essentially cell-proliferation assays, which may explain why they preferentially yield hits inactive on gametocytes, and why they rarely identify hits active on the nondividing asexual ring stages. ABS screens may only be picking the 'low hanging fruit' associated with cell-proliferative processes. Alternatively, the core biological functions ensuring gametocyte viability could largely be overlapping with that needed for the

Table 1. Summary of the TCP and target/MoA of historical and current front-runner and development pipeline compounds

Drug/compound	Class/scaffold	Target/MoA[a]	MoR[b]	Phase[c]	TCP(s) targeted	FC *in vitro* TCP5/1[d]	Refs
MMV183	Pantothenate	*Pf*ACS	*pfaccoas* (Pf3D7_0627800)		1 and 5	(12)	[84]
SJ733	Tetrahydroisoquinoline	*Pf*ATP4	*pfatp4* (Pf3D7_1211900)		1 and 5	1.7/(33)	[69]
Ciparmagin (KAE609)	Spiroindolone	*Pf*ATP4	*pfatp4* (Pf3D7_1211900)		1 and 5	1.9/(5)	[69,96]
Artefenomel (OZ439)	Endoperoxide	Unknown, heme detoxification?	Unknown		1 and 5#	2.2/(1)	[19,69]
M5717 (DDD498)	Quinoline-4-carboxamide	*Pf*EF2	*pfeef2* (Pf3D7_1451100)		1, 4, and 5	0.4/(5)	[19,57,69]
MMV390048	2-Aminopyridine	*Pf*PI4K	*pfpi4k* (Pf3D7_0509800)		1, 4, and 5	1/(6)	[69,97]
P218	Pyrimidine	*Pf*DHFR	*pfdhfr* (Pf3D7_041720)		1, 4, and 5#	(1)	[17]
Ganaplacide (KAF156)	Imidazolopiperazine	Protein secretory pathway	*pfcarl* (Pf3D7_03219000), *pfugt* (Pf3D7_1113300); *pfact* (Pf3D7_1036800)		1, 4, and 5	(1)	[98]
Lead compounds							
Methylene blue	–	Unknown	Unknown		1 and 5		–
Epoxomicin, thiostrepton	–	Proteasome	Unknown		1 and 5		–
BRD1095	Bicyclic azetidine	*Pf*aatRS (phe)	*PfpheRS* (Pf3D7_0109800)		1 and 5		[56]
DDD01035881	Sulfonamide	Pfs16	Unknown		5 #		
TM2-115	–	Putative HMT	Unknown		1 and 5		
ML324	Benzamide	Putative JMDM	Unknown		1, 4, and 5		[45]

[a]Abbreviations: ACS, acetyl CoA synthetase; ATP4, plasma membrane P-type ATPase; DHODH, dihydroorotate dehydrogenase; DHFR, dihydrofolate reductase; EF2, elongation factor 2; HMT, histone methyltransferase; JMJD, Jumonji C domain-containing histone demethylase; MoA, mechanism of action; PI4K, phosphatidylinositol-4-kinase; TCP, target candidate profile.

[b]MoR,mode of resistance, taken from Luth *et al.* [74], Cowell *et al.* [71], Duffey *et al.* [99]. PlasmoDB (www.plasmodb.org) codes for each gene provided.

[c]Discovery, ; preclinical , ; human volunteers, ; human exploratory , ; *, halted; #, gamete/oocyst targeted.

[d]Difference between asexual bloods stage pIC_{50} and normalized $pIC_{50prevalence}$ according to Dechering *et al.* [69] or if not available, in brackets - fold change (FC) of IC_{50} sexual stages (stage V gametocyte or gamete data)/IC_{50} asexual stages.

survival of an asexual stage but not with that needed for its proliferation. The inactivity of ABS hits against gametocytes therefore suggests that many essential functions in differentiated gametocytes are not easily targetable/identifiable in cell-proliferation-based assays. In this regard, it is worth noting that *Plasmodium* is the only genus of the hemosporidia (a large class of vector-borne protozoan blood parasites) that evolved the ability to undergo indefinite additional asexual schizogonies in the blood, a site where all related hemosporidia produce only gametocytes [76]. This may support the idea that gametocyte biology represents the core biology of *Plasmodium* blood-stage development associated with the normal oxidative metabolism [77]. By contrast, the processes required for asexual multiplication, including fermentative metabolism [78], developed for this unique evolutionary adaptation.

A key question is therefore what approach should be taken to identify novel agents with transmission-blocking capabilities through phenotypic screening campaigns. In a nonscreening strategy, ABS early leads would be profiled for transmission-blocking activity, with the main driver being the curative activity of the compound and transmission blocking as an asset. However, most dual-active compounds do lose between fourfold and tenfold or more of their activity against the transmissible stages compared with that seen against ABS (Table 1, [69]). Nonetheless, if dual actives are used at their minimal anti-ABS parasiticidal concentration (~3 × IC_{90}), relevant to the *in vivo* physiological concentration, they might still have a noticeable impact on transmission.

The alternative strategy of assessing selectivity straight from parallel dual frontline HTSs requires that at least two assays, on asexual and on sexual blood stages, are performed, ideally on vast compound libraries. This exercise has been conducted only on one occasion on a large scale with a library containing 68 000 compounds [79]. The assays that measure activity on gametocytes and on ABS are intrinsically different and this makes setting a threshold for stage selectivity to some extent arbitrary. That said, strategies driven by either parallel screening of diversity sets on multiple life cycle stages of *P. falciparum* [45,79], or driven by either gametocytocidal [26] or gametocidal [79] activities (Box 1) as primary screening filter, have removed selection bias and indicated several chemotypes with transmission-blocking activity. Such screens driven by a so-called selectivity towards sexual stages have effectively revealed novel chemical scaffolds that had not been identified in ABS primary screens, implying that the ~7 million compounds available to the community can still be explored for transmission-blocking potential.

In the quest for novel, transmission-blocking agents, a core issue is whether a critical minimal size or content diversity of compound libraries matter. On the former, it should be noticed that gametocyte HTSs conducted so far have interrogated altogether 300000 compounds from ABS unbiased libraries [10], which is roughly 5% of the number screened for activity against ABS parasites [80]. It is difficult to predict whether gametocyte HTSs will yield fractions of dual-active and of stage-specific compounds comparable with those obtained in ABS HTSs. The limited information from the gametocyte HTSs indicates that dual-active hits are frequently identified whereas those with a higher potency (<fivefold lower IC_{50}) in sexual versus asexual stages are rare [19,24,45].

It is thus conceivable that future transmission-blocking drug discovery will have to rely also on simplified but powerful HTS pipelines, in which one fast and easy frontline assay (e.g., viability on either stage V gametocytes or gametes, Box 1) interrogates large and chemically diverse libraries (>100k compounds). Validated hits (IC_{50} <1 μM) will then need to progress through a minimal set of **orthogonal** validation, cytotoxicity and stage-specificity assays to identify those

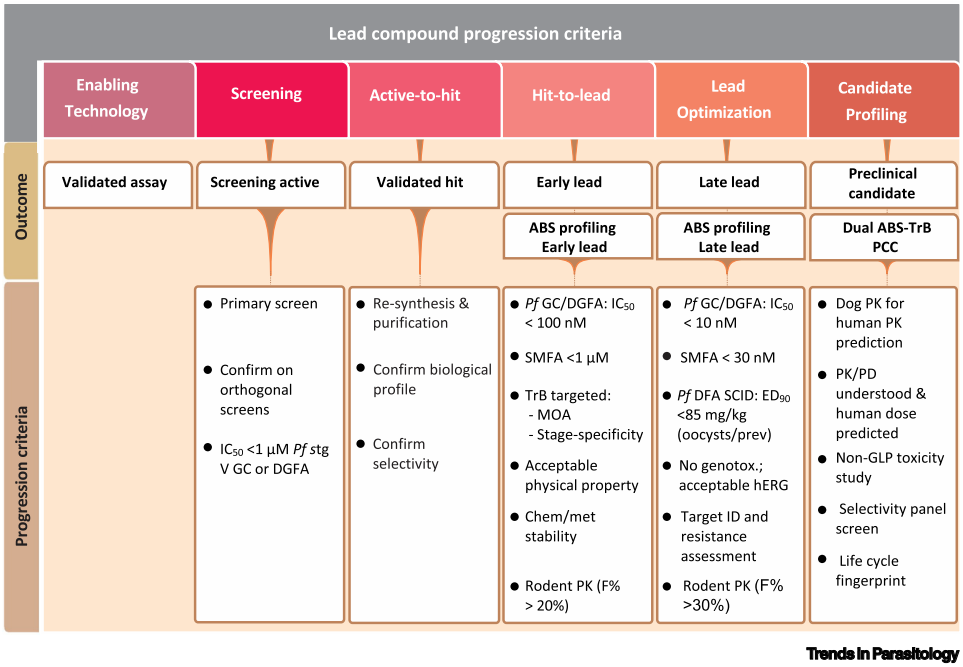

Figure 2. Transmission-blocking activity hit and lead progression criteria. Selection criteria and thresholds are proposed for transmission-blocking activity of compounds identified as hits either from transmission-blocking-driven primary screens, or as a nonscreening profiling of asexual blood stage (ABS) actives. Abbreviations: CMC, chemistry, manufacturing, and control; DGFA, dual gamete formation assay; GC, gametocyte; hERG, human ether-à-go-go-related gene; MoA, mechanism of action, PCC, pre-clinical candidate; PD, pharmacodynamics; *Pf, Plasmodium falciparum; PfDFA SCID, direct-feeding assay on humanized mouse model*; PK, pharmacokinetics;SMFA, standard membrane feeding assay; TrB, transmission-blocking.

for the final step of phenotypic confirmation (Figure 2). Since the latter is crucially dependent on gamete and/or oocyst formation, early leads would be endorsed with a potency below 100 nM in the **dual gamete formation assay** (**DGFA**) and 1 µM in the **standard membrane feeding assay (SMFA)**. At this point, establishing the MOA and having acceptable physicochemical properties would be advantageous.

Today, the search for compounds which simply block transmission remains scientifically compelling but is pragmatically not prioritized by drug-discovery and development groups like Medicines for Malaria Venture (MMV) above the discovery of compounds with additional ABS activity. This is partly because of the urgency of an impact for clinical treatment of malaria, but also since developing pure transmission-blocking compounds is complex and still requires a clear definition of a specific path from a regulatory endpoint. Proof-of-concept in measuring transmission-blocking activity in direct feeding on human volunteers [81] indeed would contribute to fill this gap. In addition, there is a need for a wide tolerability and safety margin, given that the drug would not alleviate malaria symptoms but block gametocyte transmission to prevent spreading of (potentially emerging resistant) parasites amongst an individual and her/his community. Unlike most viral diseases, immunity against malaria is not long lasting if not stimulated regularly [82]. Consequently, those living in malaria-endemic areas may become infected several times per year, and thus this burden can be relieved by interrupting transmission. This is the case with the WHO-recommended use of low-dose primaquine in areas of artemisinin resistance to block transmission, given its highly potent activity on gametocytes [83]. Should the transmission-blocking compound demonstrate equipotency against ABS parasites, as was the case for MMV183,

investigated due to its potency against gametocytes [84], the situation would be different. Undeniably, the resulting dual activity would be a clear plus for the further development of a dual combination with such a compound.

Metabolic stability should be acceptable and the bioavailability (F%) in rodents should be >20%. The medicinal chemistry plan should then produce late leads with high potency in DGFA and SMFA (IC_{50} <10 nM and 30 nM, respectively). A first proof of efficacy by blocking transmission along direct feed of mosquitos on infected humanized (NSG) mice will also be necessary to validate a late lead, as well as the absence of toxicity (genotoxicity, cardiotoxicity), with further improvements in bioavailability (F% >30%) required. Once reaching the preclinical candidate declaration point, the compound would be expected to show a clear and understandable **pharmacokinetic/pharmacodynamic** (**PK**/**PD**) relationship and a predicted dose in humans based on human PK prediction. At this stage a humanized mouse model infected by *P. falciparum* parasites could be used to approximate the efficacious dose in humans by directly feeding mosquitoes and assessing transmission-blocking efficacious exposure *in vivo.* Instead, a pragmatic approach would be to rely on the predicted efficacious concentration on ABS parasites in humans, and then assess transmission-blocking potency by SMFA. Therefore, the dose of the dual-active compound will be decided based on its ABS efficacy and its transmission-blocking efficacy measured as an observational exercise that will inform on how its potential impact could be predicted at a population level.

Outstanding questions

Which *in vitro* activities of transmission-blocking compounds translate to good activity *in vivo*?

How could the human volunteer infection model be used to increase translational evaluation of transmission-blocking efficacies?

What efficacies are required of compounds with polypharmacology to allow further development for transmission-blocking activity?

Will multistage-active compounds without equipotent activity ever be developed for transmission blocking if equipotency cannot be attained?

What would be the optimal combination of ABS and transmission-blocking candidates to protect the ABS activity from resistance transmission?

Concluding remarks

More than ever, the antimalarial community has made available a battery of techniques, assays, and strategies that enable the identification of novel chemotypes exhibiting transmission-blocking activity. Evidence clearly indicates that any treatment needs to target more than one biological process, with significant advantages associated with targeting the minor parasite population responsible for transmission. Today, low-dose primaquine is the only solution proposed to counter transmission, and this defines how next-generation improved medicines should be differentiated.

The recent discovery of novel compounds with transmission-blocking activities provides mounting evidence to optimize the functionalities such compounds would need to exhibit (see Outstanding questions). An optimal TCP-5 should favor compounds with a long plasma half-life; ability to target stage V gametocytes; ability to target immature gametocytes with, in addition, ABS activity to prevent seeding of stage V gametocytes; and efficacy in the (mosquito) vector. Development of such compounds should also address which *in vitro* activities translate to good activity *in vivo*, and the controlled human malaria infection model may indeed be useful to provide a clinical evaluation pathway. Dual-active antimalarials that are able to target ABS and have transmission-blocking activity still pose the most enticing possibilities to be used both therapeutically and for minimizing the parasite population spread. Equipotency would indeed be favored; if a dual-active compound's ABS activity is significantly more potent than the transmission-blocking activity, it will be challenging to retain sufficient room to increase the dose for the latter activity since this will be faced with constraints placed on the compound's absolute dose size, therapeutic margin, CMC (chemistry, manufacturing, and control) and development costs. However, the correct combination of ABS and transmission-blocking candidates with differential targets and activity profiles will be an important contributor to prevent resistance transmission. Compounds that display polypharmacology – targeting different proteins/processes in different stages of parasite development – indeed remain enticing and an alternative to prevent resistance development. The future should see more compounds in development with transmission-blocking activity to better support and expediate the malaria eradication agenda.

Acknowledgments

We thank Drs Jeremy Burrows and Timothy N.C. Wells (MMV) for expert discussions and proofreading and Rob Hooft van Huijsduijnen for final editing of the manuscript. We thank Dr Christian Stutzer (University of Pretoria) for assistance with the graphical elements. L-M.B. acknowledges funding from the South African Department of Science and Innovation (DSI) and the National Research Foundation (SA NRF) South African Research Chair Initiative (SARChI, UID 84627). The Institute for Sustainable Malaria Control acknowledges the South African Medical Research Council as a Collaborating Centre for Malaria Research. L-M.B. (UID 113126) and P.A. acknowledge drug discovery funding from the Italy/South Africa Joint Research Program (ISARP) from the SA DSI and NRF and the Italian Ministries of Health and of Foreign Affairs and International Cooperation. The ISARP project to P.A. is an Italy's activity in the scope of the Europe & Developing Countries Clinical Trials Partnership – EDCTP2.

Declaration of interests

D.L. is an employee of the Medicines for Malaria Venture. The authors declare no other competing interests.

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